Opened Dec 30 20 02
Décachetée le

Commissioner of Patents
Commissaire des brevets

In presence of examiner
en présence de l'examinateur

Christel G... ... NOTARIS TE DRONGEN

This is EXHIBIT FIERS-9
to
the Affidavit of Walter C. Fiers
sworn before me
this 13th day of November, 2001

Commissioner for Oath or Notary Public

KOLONIEHYBRIDISATIE

Schema

Plasmid-DNA
↓
Restrictiedigest
↓
PAGE (fragment ~250 6 %
~750 4 %)
↓
fragment eluren
↓
nick translatie
↓
koloniehybridisatie.

(1) (-1) 76T2 (band 4) fragment ← G Volckaert.

nick-translatie

100 µl α ^{32}P dATP drooggedampen (NEN ... EL
15 µl 2 x NT buffer
10 µl DNA-oplossing (76T2, ± 0.5 µg)
2,5 µl dCTP
dGTP
dTTP
} 400 µM elk
1,5 µl st H_2O
⟹ 0,5 µl DNase I (0.1 ng/µl)

10 min 37°C
ONM. op ijs.

0-staal (0.5 µl)

+ 0.5 µl DNA polymerase I (5U/µl)
Boehringer
14 à 15°C

staaltjes om 15 min
(0.5 µl) 30 min
60 min
90 min
120 min
150 min

30 µl (stock)

↳ nemen met 0.5 µl micro-cap
spotten op DEAE-32 papier → was in 0.5 M KH_2PO_4
0.5 M KH_2PO_4
H_2O
EtOH

Denhardt-solution

- 0.02 % Ficoll
- 0.02 % polyvinylpyrrolidone
- 0.02 % BSA

20x stock → samples invullen

SET

- 0.15 M NaCl
- 0.03 M Tris.Cl pH 8
- 0.001 M EDTA

[illegible]x stock

→ 3 vi fracties, splitsen op GF/C
drogen.
tellen in PPO/POPOP

↓

tot ± 70 . 10^6 tellen inkorporatie

Colony-hybridisatie

1. filter dd 3/3/77 preinkuberen in { 5 x Denhardt / 1x SET } SN 65°C
in petriplaat, zet schuddend 2 hr

2. Rinse { 5 x SET / 1 x Denhardt / 0.5 % SDS } 15 min

(SDS zet toevoegen vóór Denhardt solution – 1x)

in petriplaat, zet schuddend

3. x 20 x 20 plaat → filter na afdruppen erop leggen
dunnige extinkelen
tweede glasplaat erop en toeklemmen.

* PROBE DENATUREREN (10 000 → 10^6 tellen) 5 min 100°C

↳ NA G-50 Sephadex

toevoegen aan 7 à 10 ml Rinse-oplossing

↳ gieten in spuit → tss glazen platen brengen

hybridisatie ON 68°C
(zelf hangen)

4. { 1e WAS 25 ml { 2 x SET / 0.5 % SDS } 4 hr 65°C
in petriplaat
2e WAS id. 2 hr 65°C

zet schuddend

—

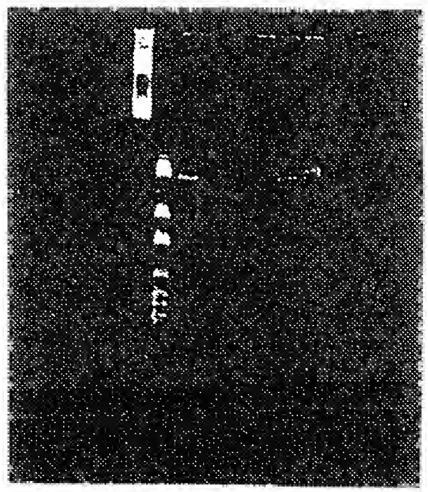
5. wassen in 3 ml Tris HCl ON [illegible]
OVERNIGHT
[illegible] schudden
([illegible])

kweken (20 ml) opgegroeid → keuze op basis van kolonies

gekozen kloons
* positief - b 9, 10
  c 9
  h 2
- kloons 1'/4 : c 1
  2'/12 : a 5
* negatief - a 8
  c 4
* twijfelachtig - d 4
  - g 8, 9

11/1 Gevolgde procedure

- cellen afcentrifugeren 5000 tpm 10 min Sm 24.
  hersuspenderen in 5 ml TES . wassen
  5000 tpm 10 min Sm 24
- hersuspenderen in 1 ml | 50 mM Tris pH 8
  | 10 % sukrose
  + 0,1 ml 10 mg/ml lysozyme in 25 mM Tris pH 8
  5 min kamertemperatuur (20 min op ijs)
- + 0,1 ml 25 mM EDTA pH 8
  + 1 ml TLM 1 0.2 % TX 100
  50 mM Tris pH 8
  25 mM EDTA
  zacht mengen
  15 min kamertemperatuur (niet langer)
- 30 min 17.000 tpm SS 34 - clearing spin
- SN + 3 ml phenol (equil) goed schudden
  10 min 5000 tpm SS 34
  2x etherextractie → verwijderen van TX100 (niet laten meeslepen bij het digereren)
  : dus uitroeien
- + 1 vol isopropanol → -80 °C
- 30 min HB4 9000 tpm - pellets drogen
  heropnemen in 1/100 TE 200 µl



Southern, gedaan dr Rik

2'/12 A5 blijkt positief
1'/4 C1 echter opnieuw negatief
(cf resultaten van kolonie-hybridisatie

Er is toch hybridisatie dw [pBR → <u>STERK</u>
(4 ng)
[pSTNV → lagere banden

11/1/'8 Sau 3A digest van { pBR 322 (4 μg)
STNV-1 (4 μg)

f -2
f -3
h -2

mengsels van telkens 30 μl met telkens 4 μg, zij ± 2,5 μg DNA

dit materiaal werd geprecipiteerd
Het is immers uit onderzoek dat het materiaal of 22 mg (Tx100) zou kunnen hebben → ext inhibitie vd restrictie-digestie.

30 μl DNA-mengsels
3,5 μl 10 x afbraak
2 μl Sau 3A. I
35,5 μl

2 hr 37°C

+ 1 μl (RNase A (0,5 μg/λ)

20 min 37°C

+ 3 μl NaAc pH 5,1 2M
+ 80 μl EtOH →↑ -20°C → afdraaien
agarosegel : 1,7 %

Verdere aanrijking vh Sau3A 28K - fragment : 4,5% TEB - PAGE. 27

Band uitsnijden
DNA elueren ~ 100 cm telling (50 cm counter)
1 M NaCl
kolomhybridisatie tov F.S. filter.

i.o.v. 50 μg/ml calf thymus sonicated DNA.

Zeer goed resultaat! 28

Konstruktie van nieuwe probes

(5) 26K - Sau 3A - fragment

Zie hiervoor
Digest
2x 4,5 % PAGE (TEB) (2 x 30 μg).

(6) IF-8 - Hinf I - fragment

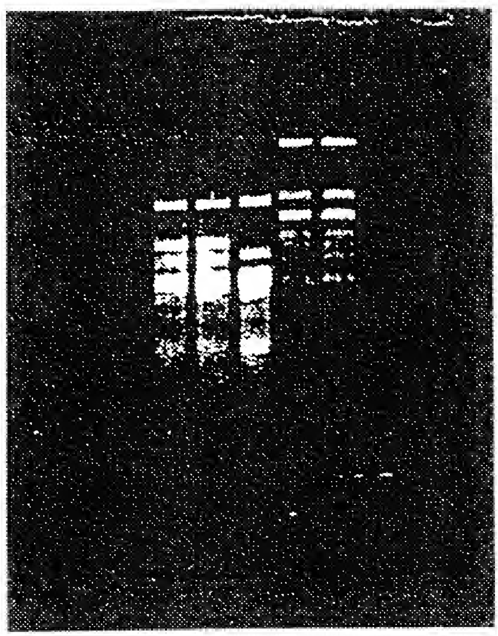
2x
20 μl DNA (~30 μg)
65 μl st H_2O
10 μl 10 x after
05 μl Hinf I (2000 U/ml)
100 μl totaal

IF-5 - Hinf I / Msp I - fragment

20 μl DNA (~30 μg)
60 μl st H_2O
10 μl 10 x after
05 μl Hinf I (2000 U/μl)
5 μl Msp I (1000 U/ml)
100 μl

4 uur 37 °C

→ 2,2 % agarose - gel (uittesten of digest volledig is)



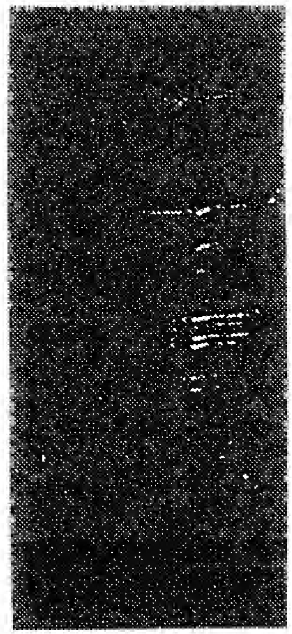
Hinf I   Hinf I.

↳ preparatieve gels

a) IF-8 : 6% PAGE (TEB)
250 V overnacht

IF-5 : 4,5% PAGE (TEB)
250 V overnacht

1631 bp
396
344
298
221/220
154
75



622
~ 350
316
291
181
147 146

elutie in 1M NaCl
precipitatie met 0.2 M NaAc pH 5.1
1 vol isopropanol

heropgenomen in IF-8 1/10 STE
IF-5 : 1x ajho → het fragment met de linkerstaart loopt samen met het intern fragment.

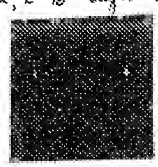
knippen met Hha I ~ 20 nucl. vh fragment met staart afgeknipt
→ gedaan

21,5 µl DNA IF-5
2,5 µl 10x ajho
1 µl Hha I. (1200 U/ml)

2 hr 37°C.

Indrogen in savant desiccator.

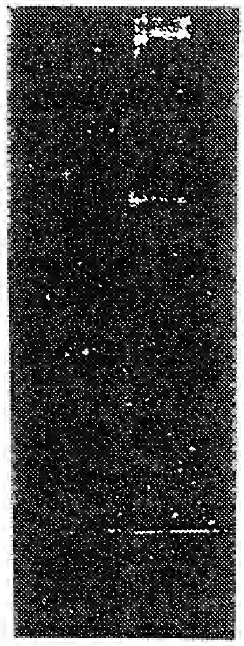
2,2% agarose testgel



digest met CK
(methylatie ?)

6) beide banden op 6% PAGE (TEB) (overnacht 25 V)

IF-8 IF-5



→ waarschl. identiek overlappingsverschijnsel

banden uitsnijden
elueren met 1M NaCl

4x IAA extraktie
1x ether extraktie
EtOH precipiteren

⟹ IF-8 = interferon-specifiek

Nick-translatie van IF-8 Hinf - fragment

(1) 50 µl α^{32}P-dATP (2-1000 mCi/mM) (1 mCi/ml) — 25 jan '80

15 µl 2 x NT buffer
5 µl DNA - oplossing
2,5 µl d GTP
CTP
TTP

7 µl dt H_2O
0,5 µl pol I (E coli)

30 µl

staalname na : 0 min
- 15
- 30

② 50 µl α ³²P dCTP drooglamper (2-3000 Ci/mMol)
(1 mCi/ml) — 1 febr '80

15 µl 2 x NT buffer
5 µl DNA oplossing
2,5 µl d G TP
ATP
TTP
7 µl st H_2O
0,5 µl DNA polymerase

30 µl

stalname na 0
15
30
45 min

↓

Sephadex G-50

Analytische staaltjes op 6% PAG (TEB) 20 x 20 cm
5 hr aan 300V

① nick-translatie α ³²P dATP 29 zeer goed resultaat

② nick-translatie α ³²P dCTP 50

Kolonie-hybridisaties

① · nick-transl ① : 100.000 tellen Cerenkov
· G_1-filter (= FS /12-14)

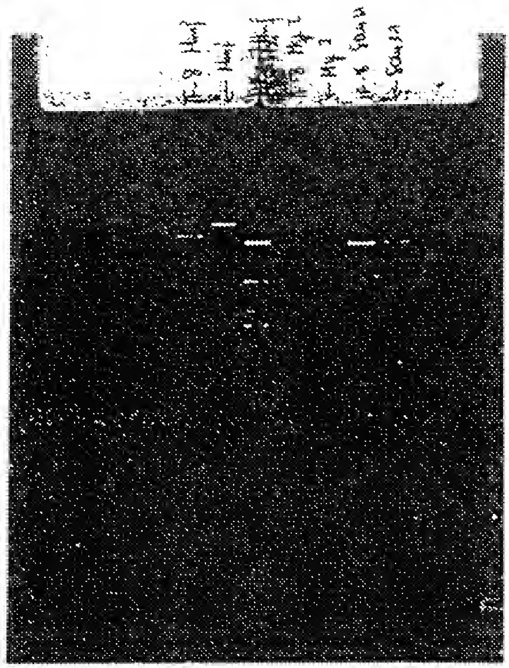
↳ kontrole op f. 11 = positief
kloon op d. 7 = zeer positief 31
Background zeer laag!

② · nick-transl. v. pTS 7 dus. I fragment a/ nick-transl. vóór fragm. isolatie
b/ fragm. isol. vóór nick-transl.

⟶ materiaal is te oud (1,5 maand!)

③ · nick-transl. ② · 50.000 tellen Cerenkov
· { G_1-filter (= FS /12-14)
  G_2-filter (= 17)

↳ ; G_1 : cf. ① . niemand zal veel niet ....



Klonenanalyse van G_1-d7

20 ml LB/tet kultuur ON opgroeien

lyseprocedure of 11/1/80

na EtOH precipitatie: materiaal heropnemen in 200 µl st H_2O (geen EDTA)

- RNase digest: + 10 µl Pancreas RNAse (0,5 µg) 20 min 37°C
- fenoliseren, etherextractie
- + 50 µl LaAc 0,2 M in st H_2O → 5 min ijs

afcentrifugeren in Eff centrifuge
SN afnemen
$\boxed{\text{niet drogen}}$!

- heropnemen in 250 µl 10 mM EDTA pH 8 (gellet lost niet zo gemakkelijk op)
- + 25 µl 0,2 M NaAc pH 5,1
+ 500 µl EtOH

restrictie-digesten

	DNA opl		10 x ophos	st H_2O	enzym	
IF-8	1	(~1,5 µg)	2	16	1	Hin f
IF-8	1	(~1,5 µg)	2	16	1	Msp 1
IF-8	1	(~1,5 µg)	2	16	1	Sau 3A
G_1-d7	5		2	11	2	Hin f
G_1-d7	5		2	11	2	Msp 1
G_1-d7	5		2	11	2	Sau 3A
pMS$_2$-7	1,5	(~1,7 µg)	2	15,5	1	Hin f

↓
3 uur 37°C
indampen in Savant tot ± 10 µl
↓
op ± 2% agarosegel (EB). 1 mm slots gegoten in cuvetten, eerst 50 V tot ?min, dan 100 V

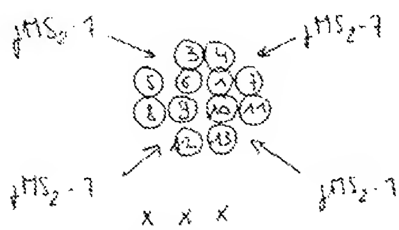
↓
overige materiaal naar Guido Volckaert.

kolonie hybridisaties (vervolg)

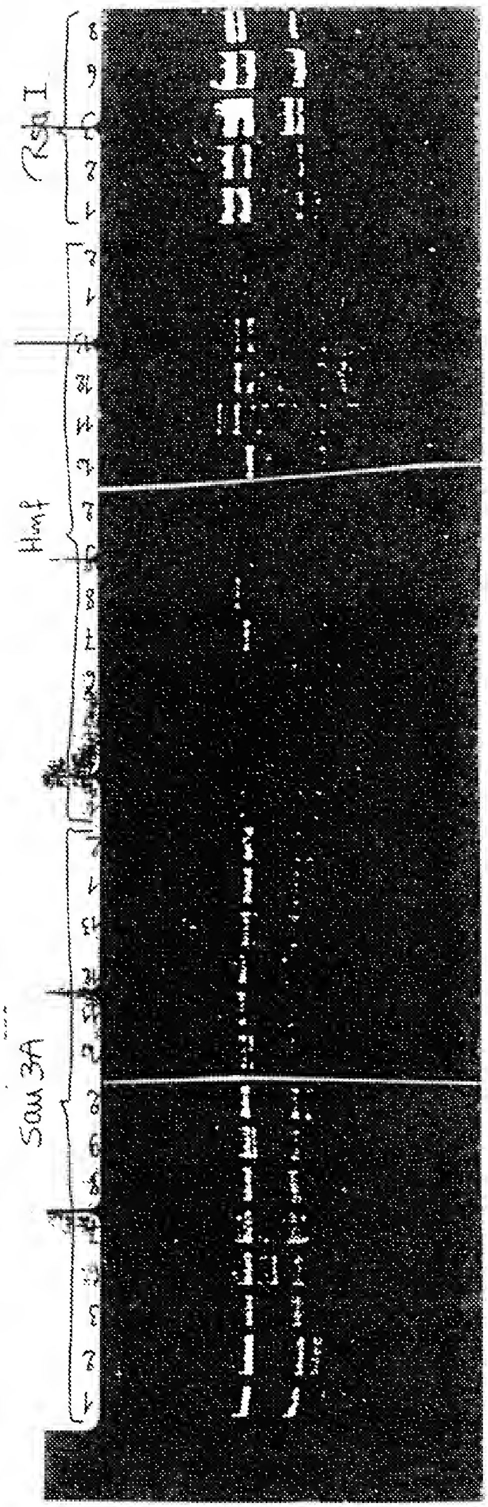
gekozen klonen

$G_1 d_7$		kloon n° 2
1'I1	d1	n°3
1'I1	f6	n°4
1'I1	g2	n°5
1'I3	d12	n°6
1'I4	a9	n°1
1'I5	e6	n°7
1'I5	g2	n°8
1'I8	b10	n°9
1'I8	e1	n°10
1'I9	g5	n°11
1'I13	f12	n°12
2'I1	b6	n°13
2'I2	a3	n°14
2'I3	f10	n°15
2'I4	g11	n°16
2'I7	b5	n°17
2'I7	c6	n°18

Alle gekozen klonen opnieuw op filter gezet : $\boxed{\text{VN-1}}$ 32

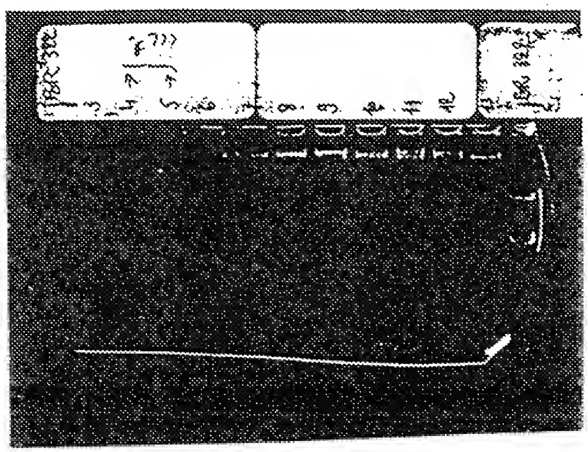


non denat. DNA van $G_1 d_7$
5' 100°C " " $G_1 d_7$
5' 100°C " " kloon 1
(telkens ± 1 μg)

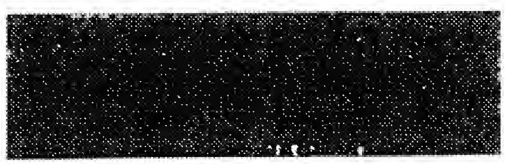

Rsa I
Hinf
Sau 3A

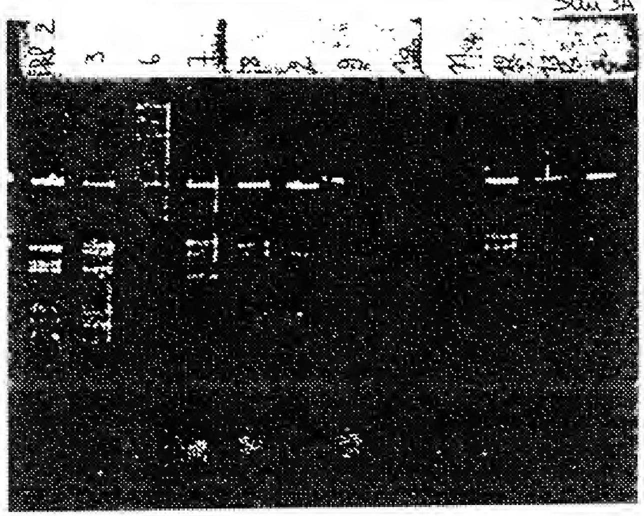
Plasm.analyse – restrictie analyse – triplicaten

20 ml LB/tet kultuur
chloramphenicol - amplificatie
Triton-lyse
↓
1% agarose gel



Restrictiedigesten





telkens 2,2% agarose en/of 5,5% acrylamide

Alle enzymatische reacties gebeuden in 30 µl bij 37°C in 1x oplos

→ 10 µl + 3 µl BFB → 2,2% agarose (wtz. 1,5%)

→ 20 µl + 6 µl BFB → 5,5% acrylamide

tryptisaties

- 2 µl poly(U)-oplossing (G. Volckaert)
  + 20 µl poly(U)-degrad. buffer { $NaHCO_3$ / Na_2CO_3 50 mM pH 9; Edta 1 mM }
  ↓
  in kapillair 2 hr 90°C
  ↓
  in Eppendorf + 20 µl 1 M NaAc pH 5.1
  + 100 µl EtOH.

- 50 λ γATP drooggedampen in Eppendorf ↓

  ↓ —— heropnemen in 20 µl 1x kinase buffer

  + 0.5 µl kinase , 25 min 37°C

- G-50 Sephadex

Kinatie van poly(U)

G-50 Sephadex

- Gel (agarose of acrylamide)

  + ~ 250 ccm Kleen → NIET OP het gel gotten!

  + 25 ml trysoakebuffer (G Valckaert).

  ½ hr schudden , kamer T.

  spoelen met trysoakebuffer (2x 5min)

  autoradiograferen

33

34

35

Digestion

(1) Cl 2 Alu I. agarose
Alu I / Msp I.
Msp I.
Rsa. I.
Ava II
Cl 1 Rsa I
Ava II.
pBR - ref.

(2) id. acrylamide

(3) Cl 2 Hinf I / Hd II. agarose
Hin d II
Hinf.
Msp I Hd I.
Pvu I / Bst N I.
Pvu I / Hae III.
pBR - ref.

(4) id. acrylamide

(5) Cl 1. Eco R II. agarose
Sau 3A.
Cl 3 Alu
Sau 3A.
Msp I.
Hinf.
Hinf - Hind I.
Eco R I
Hha I.
pBR - ref.

(6) Cl 3 id. acrylamide

(7) Cl 3 Hinf. agarose
Hind I.
Sal I
Sau 3A.
Alu

(8) id. acrylamide

9	Cl 3	Hd II. Hd II - Alu I. R II. R II - Alu I.	agarose
	Cl. 6	Hd I. Hd II - Alu I. R I. R I - Alu I.	
	Cl 7	Hd I. Hd I - Alu I. R I R I - Alu I	
	pBR. ref.		
10	Cl 3 6 7	Hd II. Hd I - Alu I. Alu I.	acrylamide
11	Cl 3 6 7	R I. R I - Alu I.	acrylamide
12	Cl 6	Alu I. Eco R II Hinf. Sau 3A	agarose
	pBR ref.		
13	Cl 8	Alu I Eco R II. Hinf Sau 3A	agarose
	pBR ref.		
14	Cl 6 8	Alu I. Eco R I. Hinf Sau 3A	acrylamide
	pBR ref		
15	Cl 1 2 3 6 8	Rsa I.	acrylamide

(17)	Cl	3, 6, 7, 8, 9	Hae III.	agarose
(18)	Cl	3, 7	Eco R I.	agarose
	Cl	3, 7	Rsa I.	
	Cl	3, 6, 7, 8, 9	~~[illegible]~~ Hae III.	
(19)	Cl	3, 6, 7, 9, 11, 13	Pvu I. Hin f. Alu I. Rsa I.	agarose
	Cl	7	Eco R II	
(20)	Cl	3, 6, 7, 9, 11, 13	Hin f.	acrylamide
(21)	Cl	7	Hind III	acrylamide
	Cl	11, 13	Rsa I.	
	Cl	3, 6, 7, 9, 11, 13	Alu I.	
(22)	Cl	3, 6, 7, 8, 9	Hind II	agarose

23	Cl	3, 6, 7, 8, 9	Pvu II.	agarose
24	Cl	3, 6, 7, 8, 9, 11.	Hind II / Pvu I.	agarose
	Cl	7	Alu I.	
25	Cl	3, 6, 7, 8, 9	Pvu I - Pvu II.	agarose
26	Cl	3	Bgl I.	agarose
	Cl	3	Bgl II - Eco R II.	
	Cl	3, 6, 7, 8, 9	Pst I.	
27	Cl	3, 6, 7	Bgl I / Hd I.	agarose
	Cl	3, 6, 7	Bgl II / Pvu I.	
	Cl	6, 7, 9, 11, 13	Hin.f / Pst I.	
28	Cl	3, 6, 7	Hae III / Hd II	agarose
	Cl	3, 6, 7	Hae III / Bgl II	
	Cl	3, 6, 7	Hd II / Pvu II	
	Cl	3	Pvu I / Pvu II	

(29) Cl 3
6
7 } Pst I / Hae III. agarose
Cl 3
6
7 } Pvu II / Hae III.

(30) Cl 3
6
7 } Pst I / Hae III. acrylamide
Cl 3
6
7 } Pvu II / Hae III.
Cl 3
6
7 } Bgl II / Hae III.

(31) Cl 13 Hd II / Bgl II. agarose 1.5%
Cl 3
6
13. } Hd II / Bgl II. 1.8%
pBR ufs

(32) Cl 3
6
7 } Bgl II. agarose.
Cl 3
6
7 } Bgl II / Pvu II.
Cl 3
6
7. } Bgl II / Hind II.
Cl 3
6
7 } Rsa I / Hind II.

(33) Cl 19
20
21. } Hae III
Cl 13 Hae III
Cl 13 Rsa I.

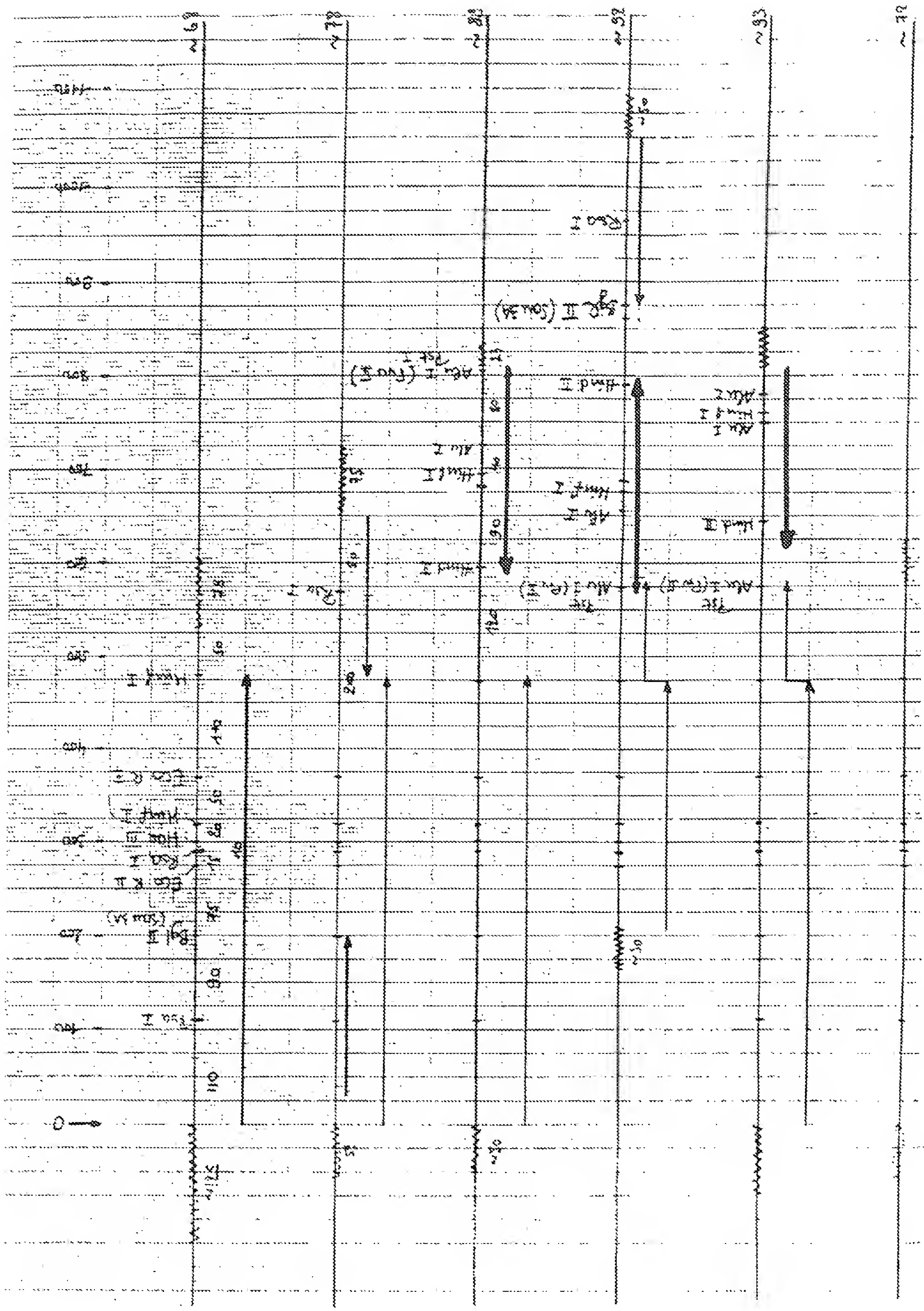
(34) Cl 6
7
21 } Hin f. agonox.
Cl 21 Eco R II.
Cl 21 Taq I.

(35) Cl 19
20
21 } Pvu I - Pvu II. agonox
Cl 19
20
21 } Rsa I.
Cl 19
20
21 } Bgl II - Hind II.
Cl 19
20
21 } Hin f.
Cl 19
20
21 } Hsp I.

(36) Cl 19 Hin f agonox
Hin f - Pvu II
Eco R I.
Taq I - Rsa.
Cl 20 Eco R II.
Taq I.
Alu I.
Cl 21 Alu I.
Cl 26K-1 Rsa I.
Bst E II
Mbo I - Rsa I.
Mbo II - Hin f
Mbo II - Sau 3A

(37) Cl 19 Rsa - Taq I. agonox
Cl 20 Rsa - Taq I
Cl 26K-1 Mbo II
Cl 26K-1 Mbo II - Hin f
Mbo II - Rsa I
Mbo II - Sau 3A

(38) Cl 20 Tag I
Cl 19 Tag I - Rsa
Cl 20 Tag I - Rsa



This is EXHIBIT FIERS-10
to
the Affidavit of Walter C. Fiers
sworn before me
this 19 th day of November, 2001

Commissioner for Oath or Notary Public

F IF
88
DRAFT

Opened Décachetée le Dec 30 20 02

Commissioner of Patents
Commissaire des brevets

in presence of examiner
en présence de l'examinateur

RECOMBINANT DNA MOLECULES AND THEIR USE IN PRODUCING STRUCTURAL GENES FOR HUMAN FIBROBLAST INTERFERON

TECHNICAL FIELD OF INVENTION

This invention relates to recombinant DNA molecules and their use in producing structural genes for human fibroblast interferon. The recombinant DNA molecules disclosed herein are characterized by DNA sequences that code for polypeptides whose amino acid sequence and composition are substantially consistent with human fibroblast interferon.

BACKGROUND ART

Two classes of interferons ("IF") are known to exist. Interferons of Class I are small, acid stable (glyco)-proteins that render cells resistant to viral infection (A. Isaacs and J. Lindenmann, "Virus Interference I. The Interferon", Proc. Royal Soc. Ser. B., 147, pp. 258-67 (1957) and W.E. Stewart, II, The Interferon System, Springer-Verlag (1979) (hereinafter "The Interferon System")). Class II IFs are acid labile. At present, they are poorly characterized. Although to some extent cell specific (The Interferon System, pp. 135-45), IFs are not virus specific. Instead, IFs protect cells against a wide spectrum of viruses.

Two antigenically distinct species of Class I human interferon ("HIF") are known to exhibit IF activity. One IF species, fibroblast interferon ("F IF"), is produced upon appropriate induction in diploid fibroblast cells. Another IF species, leukocyte interferon ("Le IF") is produced together with minor amounts of F IF upon appropriate induction in human leukocyte and lympho-

blastoid cells. Both are heterogeneous in regard to size, presumably because of the carbohydrate moiety. F IF has been extensively purified and characterized (E. Knight, Jr., "Interferon: Purification And Initial Characterization From Human Diploid Cells", Proc. Natl. Acad. Sci. USA, 73, pp. 520-23 (1976)). It is a glyco-protein of about 20,000 molecular weight (M. Wiranowska-Stewart et al., "Contributions Of Carbohydrate Moieties To The Physical And Biological Properties Of Human Leukocyte, Lymphoblastoid And Fibroblast Interferons", Abst. Ann. Meeting Amer. Soc. Microbiol., p. 246 (1978)). Its amino acid composition has been determined (E. Knight, Jr. et al., "Human Fibroblast Interferon: Amino Acid Analysis And Amino-Terminal Amino Acid Sequence", Science, 207, pp. 525-26 (1980)). Elucidation of its amino acid sequence is in progress. To date, the amino acid sequence of the NH_2 terminus of the mature protein has been reported for the first 13 amino acid residues: Met-Ser-Tyr-Asn-Leu-Leu-Gly-Phe-Leu-Gln-Arg-Ser-Ser... (E. Knight, Jr. et al., supra). Two distinct genes, one located on chromosome 2, the other on chromosome 5, have been reported to code for F IF (D.L. Slate and F.H. Ruddle, "Fibroblast Interferon In Man Is Coded By Two Loci On Separate Chromosomes", Cell, 16, pp. 171-80 (1979)).

Le IF has likewise been purified and characterized. Two components have been described, one of 21000 to 22000 and the other of 15000 to 18000 molecular weight (K.C. Zoon, et al., "Purification And Partial Characterization Of Human Lymphoblastoid Interferon", Proc. Natl. Acad. Sci. USA, 76, pp. 5601-605 (1979)). A portion of the amino acid sequence of Le IF has also been determined, i.e., 20 amino acids from the amino terminus of the mature protein (K.C. Zoon et al., "Amino-Terminal Sequence Of The Major Component Of Human Lymphoblastoid Interferon", Science, 207, pp. 527-28 (1980)). A comparison of the initial amino acid sequence of F IF and Le IF reveals no detectable homology within the first 13 amino acids. The total amino acid compositions of the two species are also distinct. In addition, degradation of the sugar residues of the two species by periodate indicates that the carbohydrate structure of the two IFs is different (M. Wiranowska-Stewart et al., supra).

The two species of HIF have a number of different properties. For example, anti-human Le IF antibodies are less efficient

against F IF and anti-sera to human F IF have no activity against human Le IF (The Interferon System, p. 151) and Le IF displays a high degree of activity in cell cultures of bovine, feline or porcine origin whereas F IF is hardly active in those cells but has been reported to be active in rat cells (P. Duc-Goiran et al., "Studies On Virus-Induced Interferons Produced By The Human Amniotic Membrane And White Blood Cells", Arch. Ges. Virus Forsch., 34, pp. 232-43 (1971)). In addition, the two IFs result from different mRNA species (and therefore from presumable different structural genes) that code for polypeptides of different primary sequence (R.L. Cavalieri et al., "Synthesis of Human Interferon by Xenopus laevis Oocytes: Two Structural Genes for Interferon in Human Cells", Proc. Natl. Acad. Sci. USA, 74, pp. 3287-91 (1977)).

Although both Le and F IFs occur in a glycosylated form, removal of the carbohydrate moiety (P.J. Bridgen et al., "Human Lymphoblastoid Interferon", J. Biol. Chem., 252, pp. 6585-87 (1977)) or synthesis of IF in the presence of inhibitors which preclude glycosylation (W.E. Stewart, II et al., "Effect of Glycosylation Inhibitors On The Production And Properties Of Human Leukocyte Interferon", Virology, 97, pp. 473-76 (1979); J. Fujisawa et al., "Nonglycosylated Mouse L Cell Interferon Produced By The Action Of Tunicamycin", J. Biol. Chem., 253, pp. 8677-79 (1978); E.A. Havell et al., "Altered Molecular Species Of Human Interferon Produced In The Presence Of Inhibitors Of Glycosylation", J. Biol. Chem., 252, pp. 4425-27 (1977); The Interferon System, p. 181) yields a smaller form of IF which still retains most or all of its IF activity.

Both F IF and Le IF may, like many human proteins, be polymorphic. Therefore, cells of particular individuals may produce IF species within each of the more general F IF and Le IF classes which are physiologically similar but structurally slightly different than the protein's as of the class of which it is a part. Therefore, while the protein structure of the F IF or Le IF may be generally well-defined, particular individuals may produce IFs that are slight variations thereof.

IF is usually not detectable in normal or healthy cells (The Interferon System, pp. 55-57). Instead, the protein is produced as a result of the cell's exposure to an IF inducer. IF inducers are usually viruses but may also be non-viral in character,

such as natural or synthetic double-stranded RNA, intracellular microbes, microbial products and various chemical agents. Numerous attempts have been made to take advantage of these non-viral inducers to render human cells resistant to viral infection (S. Baron and F. Dianzani (eds.), Texas Reports On Biology And Medicine, 35 ("Texas Reports"), pp. 528-40 (1977)). These attempts have not been very successful. Instead, use of exogenous IF itself is now preferred.

As an antiviral agent, HIF has been used to treat the following: respiratory infections (Texas Reports, pp. 486-96); herpes simplex keratitis (Texas Reports, pp. 497-500; R. Sundmacher, "Exogenous Interferon in Eye Diseases", International Virology IV, The Hague, Abstract nr. W2/11, p. 99 (1978)); acute hemorrhagic conjunctivitis (Texas Reports, pp. 501-10); adenovirus keratoconjunctivitis (A. Romano et al., ISM Memo I-A8131 (October, 1979)); varicella zoster (Texas Reports, pp. 511-15); cytomegalovirus infection (Texas Reports, pp. 523-27); and hepatitis B (Texas Reports, pp. 516-22). See also The Interferon System, pp. 307-19. In these treatments F IF and Le IF may display different dose/response curves. However, large-scale use of IF as an antiviral agent requires larger amounts of HIF than heretofore have been available.

IF has other effects in addition to its antiviral action. For example, it antagonizes the effect of colony stimulating factor, inhibits the growth of hemopoietic colony-forming cells and interfers with the normal differentiation of granulocyte and macrophage precursors (Texas Reports, pp. 343-49). It also inhibits erythroid differentiation in DMSO-treated Friend leukemia cells (Texas Reports, pp. 420-28). Some cell lines may be considerably more sensitive to F IF than to Le IF in these regards (S. Einhorm and H. Strander, "Is Interferon Tissue-Specific? - Effect Of Human Leukocyte And Fibroblast Interferons On The Growth Of Lymphoblastoid And Osteosarcoma Cell Lines", J. Gen. Virol., 35, pp. 573-77 (1977); T. Kuwata et al., "Comparison Of The Suppression Of Cell And Virus Growth In Transformed Human Cells By Leukocyte And Fibroblast Interferon", J. Gen. Virol., 43, pp. 435-39 (1979)).

IF may also play a role in regulation of the immune response. For example, depending upon the dose and time of application in relation to antigen, IF can be both immunopotentiating and immunosuppressive in vivo and in vitro (Texas Reports, pp. 357-69). In

In addition, specifically sensitized lymphocytes have been observed to produce IF after contact with antigen. Such antigen-induced IF could therefore be a regulator of the immune response, affecting both circulating antigen levels and expression of cellular immunity (Texas Reports, pp. 370-74). IF is also known to enhance the activity of killer lymphocytes and antibody-dependent cell-mediated cytotoxicity (R.R. Herberman et al., "Augmentation By Interferon Of Human Natural And Antibody-Dependent Cell-Mediated Cytotoxicity", Nature, 277, pp. 221-23 (1979); P. Beverley and D. Knight, "Killing Comes Naturally", Nature, 278, pp. 119-20 (1979); Texas Reports, pp. 375-80; M. Lucero et al., "Induction And Kinetics Of Natural Killer Cells in Humans Following Interferon Therapy", Nature, 282, pp. 417-19 (1979); S. Einhorn et al.,

Acta Med. Scand., 20, pp. 477-83 (1978)). Both may be directly or indirectly involved in the immunological attack on tumor cells.

Therefore, in addition to its use as a human antiviral agent, HIF has potential application in antitumor and anticancer therapy (The Interferon System, pp. 319-21 and 394-99). It is now known that IFs affect the growth of many classes of tumors in many animals (The Interferon System, pp. 292-304). They, like other anti-tumor agents, seem most effective when directed against small tumors. The antitumor effects of animal IF are dependent on dosage and time but have been demonstrated at concentrations below toxic levels. Accordingly, numerous investigations and clinical trials have been and continue to be conducted into the antitumor and anticancer properties of HIFs. These include treatment of several malignant diseases such as osteosarcoma, acute myeloid leukemia, multiple myeloma and Hodgkin's disease (Texas Reports, pp. 429-35). ✓ In addition, F IF has recently been shown to cause local tumor regression when injected into subcutaneous tumoral nodules in melanoma and breast carcinoma-affected patients (T. Nemoto et al., "Human Interferons And Intralesional Therapy Of Melanoma And Breast Carcinoma", Amer. Assoc. For Cancer Research, Abs nr. 993, ✓ p. 246 (1979)). Significantly, some cell lines which resist the anticellular effects of Le IF remain sensitive to F IF. This differential effect suggests that F IF may be usefully employed against certain classes of resistant tumor cells which appear under selective pressure in patients treated with high doses of

growth of many classes of tumors in many animals (The Interferon System, pp. 292-304). They, like other antitumor agents, seem most effective when directed against small tumors. The antitumor effects of animal IF are dependent on dosage and time but have been demonstrated at concentrations below toxic levels. Accordingly, numerous investigations and clinical trials have been and continue to be conducted into the antitumor and anticancer properties of HIFs. These include treatment of several malignant diseases such as osteosarcoma, acute myeloid leukemia, multiple myeloma and Hodgkin's disease (Texas Report, pp. 429-35). In addition, F IF has recently been shown to cause local tumor regression when injected into subcutaneous tumoral nodules in melanoma and breast carcinoma-affected patients (T. Nemoto et al., Human Interferons And Intralesional Therapy of Melanoma And Breast Carcinoma", Amer. Assoc. For Cancer Research, Abs nr. 993, p. 246 (1979)). Significantly, some cell lines which resist the anticellular effects of Le IF remain sensitive to F IF. This differential effect suggests that F IF may be usefully employed against certain classes of resistant tumor cells which appear under selective pressure in patients treated with high doses of Le IF (T. Kuwata et al., supra; A. A. Creasy et al., "The Role of G_0-G_1 Arrest In The Inhibition Of Tumor Cell Growth By Interferon", Abstracts, Conference On Regulatory Functions Of Interferons, ~~"Regulatory Functions Of Interferons", a~~ N. Y. Acad. Sci., ~~Abstract nr. 17~~ nr. 17 October 23-26, (1979)). Although the results of these clinical tests are encouraging, the antitumor and anticancer applications of HIF have been severely hampered by lack of an adequate supply of purified HIF.

At the biochemical level IFs induce the formation of at least 3 proteins, a protein kinase (B. Lebleu et al., "Interferon, Double-Stranded RNA And Protein Phosphorylation", Proc. Natl. Acad. Sci. USA, 73, pp. 3107-11 (1976); A. G. Hovanessian and I. M. Kerr, "The (2'-5') Oligoadenylate (ppp A2'-5A2'-5'A) Synthetase And Protein Kinase(s) From Interferon-Treated Cells", Eur. J. Biochem., 93, pp. 515-26 (1979)), a (2'-5')oligo(A) polymerase (A. G. Hovanessian

Several non-bacterial proteins (genes) have been obtained in E. coli using recombinant DNA technology.

comprises the steps of producing a single-stranded DNA copy (cDNA) of a purified messenger RNA (mRNA) template for the desired protein; converting the cDNA to double-stranded DNA; linking the DNA to an appropriate site in an appropriate cloning vehicle to form a recombinant DNA molecule and transforming an appropriate host with that recombinant DNA molecule. Such transformation may permit the host to produce the desired protein. These include, for example, Le IF (C. Weissmann et al., Seminar, Massachusetts Institute of Technology, January 16, 1980). In addition, recombinant DNA technology has been employed to produce a plasmid said to contain a gene sequence coding for F IF (T. Taniguchi et al., "Construction And Identification Of A Bacterial Plasmid Containing The Human Fibroblast Interferon Gene Sequence", Proc. Japan Acad., 55, Ser. B, pp. 464-69 (1979).

However, in neither of the foregoing has the actual gene sequence of F IF been described and in neither has that sequence been compared to the initial amino acid sequence or amino acid composition of authentic F IF. The former ~~Weissmann~~ work is directed only to Le IF, ~~which is~~ a distinct chemically, biologically and immunologically ~~from~~ Class I interferon from F IF (cf. supra). The latter ~~Taniguchi~~ report is ~~results are~~ based solely on hybridization data. These ~~latter~~ data do not enable one to determine if the selected clone contains the complete or act coding gene sequence for F IF or if ~~that~~ the cloned gene sequence will be able to express F IF ~~be expressed~~ in bacteria. Hybridization only establishes that a particular DNA insert is to some extent homologous with and complementary to a mRNA component of the poly(A) RNA that induces interferon activity when injected into oocytes. Moreover, the extent of the homology is dependent on the hybridization conditions chosen for the screening process. Therefore, hybridization to a mRNA component of poly(A) RNA alone does not demonstrate that the selected DNA sequence is a sequence which codes for F IF or a polypeptide which displays the immunological or biological activity of F IF.

DISCLOSURE OF THE INVENTION

The present invention avoids the uncertainties referred to by providing at least one recombinant DNA molecule characterized by a structural gene whose nucleotide sequence is substantially consistent with the known amino acid composition and ~~sequences~~ sequence or of authentic F IF.

By virtue of this invention, it is therefore possible to obtain a structural gene that codes for a polypeptide whose amino acid sequence and composition is substantially consistent with authentic F IF. Replication of these genes in appropriate recombinant DNA molecule-host combinations permits the production of large quantities of these genes. These genes are useful, either as produced in the host or after appropriate derivatization or modification, in compositions and methods for detecting and improving the production of these products themselves and in selecting other genes related thereto.

~~BRIED~~ BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic outline of one embodiment of a process of this invention for preparing a mixture of recombinant DNA molecules, some of which are characterized by inserted DNA sequences that characterize this invention.

Figure 2 is a schematic outline of the initial clone screening process of this invention.

Figure 3 is a schematic outline of one embodiment of a clone screening process using DNA sequences prepared in accordance with the invention.

~~Figure 4 is a restriction map of one of the clones of the invention; the absolute position of each restriction site in this clone has not been determined.~~

Figure —

others

DNA Sequence--A linear array of nucleotides connected one to the other by phosphodiester bonds between the 3' and 5' carbons of adjacent pentoses.

Codon--A DNA sequence of three nucleotides (a triplet) which encodes through mRNA an amino acid, a translation start signal or a translation termination signal. For example, the nucleotide triplets TTA, TTG, CTT, CTC, CTA and CTG encode for the amino acid leucine ("Leu"), TAG, TAA and TGA are translation stop signals and ATG is a translation start signal.

Reading Frame--The grouping of codons during translation of mRNA into amino acid sequences. During translation the proper reading frame must be maintained. For example, the sequence GCTGGTTGTAAG may be translated in three reading frames or phases, each of which affords a different amino acid sequence:

GCT GGT TGT AAG--Ala-Gly-Cys-Lys
G CTG GTT GTA AG--Leu-Val-Val
GC TGG TTG TAA G--Trp-Leu-(STOP)

Polypeptide--A linear array of amino acids connected one to the other by peptide bonds between the α-amino and carboxy groups of adjacent amino acids.

Genome--The entire DNA of a cell or a virus. It includes *inter alia* the structural genes coding for the polypeptides of the substance, as well as operator, promoter and ribosome binding and interaction sequences, including sequences such as the Shine-Dalgarno sequences.

Structural Gene--A DNA sequence which encodes through its template or messenger RNA ("mRNA") a sequence of amino acids characteristic of a specific polypeptide.

Transcription--The process of producing mRNA from a structural gene.

Translation--The process of producing a polypeptide from mRNA.

Expression--The process undergone by a structural gene to produce a polypeptide. It is a combination of transcription and translation.

<u>Plasmid</u>--A nonchromosomal double-stranded DNA sequence comprising an intact "replicon" such that the plasmid is replicated in a host cell. When the plasmid is placed within a unicellular organism, the characteristics of that organism may be changed or transformed as a result of the DNA of the plasmid. For example, a plasmid carrying the gene for tetracycline resistance (Tet^R) transforms a cell previously sensitive to tetracycline into one which is resistant to it. A cell transformed by a plasmid is called a "transformant".

<u>Phage or Bacteriophage</u>--Bacterial virus many of which consist of DNA sequences encapsidated in a protein envelope or coat ("capsid").

<u>Cloning Vehicle</u>--A plasmid, phage DNA or other DNA sequence~~s~~ which ~~are~~ is able to replicate in a host cell, characterized by one or a small number of endonuclease recognition sites at ~~shich~~ which such DNA sequences may be cut in a determinable fashion without attendant loss of an essential biological function of the DNA, <u>e.g.</u>, replication, production of coat proteins or loss of promoter or binding sites, and which contain a marker suitable for use in the identification of transformed cells, <u>e.g.</u>, tetracycline resistance or ampicillin resistance. A cloning vehicle is often called a vector.

<u>Cloning</u>--The process of obtaining a population of organisms or DNA sequences derived from one such organism or sequence by asexual reproduction.

<u>Recombinant DNA Molecule</u> or <u>Hybrid DNA</u>--A molecule consisting of segments of DNA from different genomes which have been joined end-to-end outside of living cells and have the capacity to infect some host cell and be maintained therein.

<u>Expression Control Sequence</u>--A sequence of nucleotides that controls and regulates expression of structural genes when operatively linked to those genes.

Referring now to Figure 1, we have shown therein a schematic outline of one embodiment of a process for preparing a mixture of recombinant DNA molecules, some of which include inserted DNA sequences that characterize this invention.

PREPARATION OF POLY(A) RNA CONTAINING HUMAN FIBROBLAST INTERFERON mRNA (F IF mRNA)

The RNA used in this invention was extracted from human VGS cells, a diploid fibroblast cell line which can be propagated in monolayer cultures at 37°C. ~~Interferon~~ F IF is produced in these cells on induction with poly(I,C) ~~and~~ in the presence of cycloheximide.

For a typical RNA isolation, each of 20 roller bottles of diploid VGS cells in confluent monolayer were "primed" overnight with 100 units/ml F IF and the cultures induced for 1 h with 100 μg/ml poly(I,C) and 50 μg/ml cycloheximide, incubated with cycloheximide (50 μg/ml) for 4 h, harvested by scraping into phosphate-buffered saline and spun down. The cells were lysed for 15 min at 0°C ~~and~~ by [illegible] procedures to remove the intact nuclei containing the DNA and to isolate the cytoplasmic RNA by suspending them in hypotonic buffer (10 mM Tris-HCl (pH 7.4), 10 mM NaCl and 1.5 mM $MgCl_2$) and adding NP40 to 1%. Nuclei were removed by pelleting in a Sorvall SS-34 rotor for 5 min at 3000 rpm. Sodium dodecyl sulphate ("SDS") and EDTA were added to the supernatant to 1% and 10 mM, respectively, and the mixture extracted 5 times with 2x vol. of 1:1 redistilled phenol and chloroform-isoamyl alcohol (25:1), the aqueous phases containing the RNA being separated by centrifugation in a Sorvall SS-34 rotor at 8000 rpm for 10 min after each extraction. The RNA was precipitated from the aqueous phase by addition of 1/10 vol 2 M sodium acetate (pH 5.1) and 2.5 vol ~~of~~ ethanol. Usually, 60 to 90 μg of total cytoplasmic RNA ~~was~~ typically obtained per roller bottle.

Other procedures to extract the cytoplasmic RNA have also been used. For example, the cells were totally lysed after homogenization in 0.2 M Tris-HCl (pH 9.0), 50 mM NaCl, 20 mM EDTA and 0.5% SDS ~~sodium dodecyl sulphate~~ and extracted with phenol-chloroform as above or the washed cells were suspended in 400 ul 0.1 M NaCl, 0.01 M Tris-HCl (pH 7.5), and 0.001 M EDTA ("NTE buffer") and 2.5 ml 4 M guanidinium-isothiocyanate and 1 M ß-mercaptoethanol in 20 mM sodium acetate (pH 5.0) were added and the cells homogenized. The lysate was layered on a 1.3-ml 5-7 M CsCl cushion in a Beckman SW-60 Ti nitrocellulose tube, spun for 17 h at 39000 rpm to pellet the RNA and separate it from DNA, proteins and lipids and the RNA extracted once with phenol-chloroform (F.H. Reynolds et al., "Interferon Activity Produced by Translation of Human Interferon Messenger RNA in Cell-Free Ribosomal Systems and In Xenopus Oocytes", Proc. Natl. Acad. Sci. USA, 72, pp. 4881-87 (1975); J. Mosher et al., "Characterization of Interferon Messenger RNA from Human Lymphoblastoid Cells", J. Gen. Virol., 44, pp. 231-34 (1979)).

The total RNA was assayed for the presence of F IF mRNA by injection into the cytoplasm of Xenopus laevis oocytes and determination of the F IF ~~interferon~~ activity induced therein (Reynolds et al., supra). The assay was conducted by dissolving the RNA in water and injecting about 50 ul into each oocyte. The oocytes were incubated overnight at room temperature in Barth medium (J. Gurdon, J. Embryol. Exper. Morphol., 20, pp. 401-14 (1968)), homogenized in part of the medium, the debris removed by centrifugation, and the F IF activity of the supernatant determined. Detection of F IF activity was by reduction of virus-induced cytopathic effect (W. E. Stewart and S. E. Sulkin, Proc. Soc. Exp. Biol. Med., 123, pp. 650-53 (1966)). The challenge virus was vesicular stomatitis virus (Indiana

strain) and the cells were human diploid fibroblasts trisomic for chromosome 21 to afford higher F IF sensitivity. F IF activity is expressed relative to the IF reference standard 69/19.

Poly(A) RNA containing F IF mRNA was isolated from the cytoplasmic RNA by adsorption to oligo(dT)-cellulose (type 7; P-L Biochemicals) in 0.4 M NaCl, 10 mM Tris-HCl, (pH 7.8), 10 mM EDTA and 0.2% SDS ~~sodium dodecyl sulphate~~ for 10 min at room temperature. RNA aggregation was minimized by heating the RNA for 2 min at 70°C prior to adsorption. After washing the cellulose with the above-mentioned buffer, the poly(A) RNA fraction was eluted with 10 mM Tris-HCl (pH 7.8), 1 mM EDTA and 0.2% SDS. ~~sodium dodecyl sulphate.~~ It usually comprised 4-5% of the total RNA, as measured by optical density at 260 nm.

A further purification to enrich the poly(A) RNA in F IF mRNA was effected by formamide-sucrose gradients (T. Pawson et al., "The Size of Rous Sarcoma Virus mRNAs Active in Cell-Free Translation", Nature, 268, pp. 416-20 (1977)). These gradients gave much higher resolution than ~~the~~ non-denaturing sucrose gradients. Usually about 80 μg poly(A) RNA was dissolved in 50% formamide, 100 mM LiCl, 5 mM EDTA, 0.2% SDS ~~sodium dodecyl sulphate~~ and 10 mM Tris-HCl (pH 7.4), heated at 37°C for 2 min to prevent aggregation and loaded on a 5-20% sucrose gradient in a Beckman SW-60 Ti polyallomer tube. After centrifugation at 20°C for 4 1/2 h at 60000 rpm in the Beckman SW-60 Ti rotor with total ^{14}C-labeled eukaryotic RNA serving as size markers, the gradient was fractionated and the optical density of the fractions ~~was~~ determined. All RNA fractions were precipitated twice with 0.5 M NaCl and 2.5 vol ethanol and assayed for interferon mRNA activity as described above.

Alternatively, the oligo(dT)-adsorbed mRNA (60 μg) was fractionated by electrophoresis in a 4% polyacrylamide gel in 7 M urea, 0.1% SDS; ~~sodium dodecyl sulphate,~~

CLONING OF DOUBLE-STRANDED DNA

A wide variety of host/cloning vehicle combinations may be employed in cloning the double-stranded cDNA prepared in accordance with this invention. For example, useful cloning vehicles may consist of segments of chromosomal, non-chromosomal and synthetic DNA sequences, such as various known derivatives of SV40 and known bacterial plasmids, e.g., plasmids from E. coli including col E1, pCR1, pBR322, pMB9 and their derivatives, wider host range plasmids, e.g., RP4, phage DNAs, e.g., the numerous derivatives of phage λ e.g., NM 989, and other DNA phages, e.g., M13 and fd, and vectors derived from combinations of plasmids and phage DNAs such as plasmids which have been modified to employ phage DNA or other expression control sequences or yeast plasmids such as the 2 μ plasmid or derivatives thereof. Useful hosts may include bacterial hosts such as E. coli HB 101, E. coli X1776, E. coli X2282, E. coli MRCI and strains of Pseudomonas, Bacillus subtilis, Bacillus stearothermophilus and other bacilli, yeasts and other fungi, animal or plant hosts such as animal (including human) or plant cells in culture or other hosts. Of course, not all host/vector combinations may be equally efficient. The particular selection of host/cloning vehicle combination may be made by those of skill in the art after due consideration of the principles set forth without departing from the scope of this invention.

Furthermore, within each specific cloning vehicle, various sites may be selected for insertion of the double-stranded DNA. These sites are usually designated by the restriction endonuclease which cuts them. For example, in pBR322 the PstI site is located in the gene for β-lactamase, between the nucleotide triplets that code for amino acids 181 and 182 of that protein. This site was employed by C. Weissmann et al., supra, in their synthesis of polypeptides displaying an immunological or biological activity of LeIF. One of the two HindII endonuclease recognition sites is between the triplets coding for amino acids 101 and 102 and one of the several Taq sites at the triplet coding for amino acid 45 of β-lactamase in pBR322. In similar fashion, the EcoRI site and the PvuII site in this plasmid lie outside of any coding region, the EcoRI site being located between the genes coding for resistance to tetracycline and ampicillin, respectively. This site was employed by T. Taniguchi et al., supra, in

their recombinant synthetic scheme. These sites are well recognized by those of skill in the art. It is, of course, to be understood that a cloning vehicle useful in this invention need not have a restriction endonuclease site for insertion of the chosen DNA fragment. Instead, the vehicle could be joined to the fragment by alternative means.

The vector or cloning vehicle and in particular the site chosen therein for attachment of a selected DNA fragment to form a recombinant DNA molecule is determined by a variety of factors, e.g., number of sites susceptible to a particular restriction enzyme, size of the protein to be expressed, susceptibility of the desired protein to proteolytic degradation by host cell enzymes, contamination of the protein to be expressed by host cell proteins difficult to remove during purification, expression characteristics, such as the location of start and stop codons relative to the vector sequences, and other factors recognized by those of skill in the art. The choice of a vector and an insertion site for a particular gene is determined by a balance of these factors, not all selections being equally effective for a given case.

Although several methods are known in the art for inserting foreign DNA into a cloning vehicle or vector to form a recombinant DNA molecule, the method preferred in accordance with this invention is characterized by digesting the plasmid (in particular pBR322) with that restriction enzyme specific to the site chosen for the insertion (in particular PstI) and adding dA tails to the 3' termini by terminal transferase. In similar fashion, the double-stranded cDNA is elongated by the addition of dT tails to the 5' termini to allow joining to the tailed plasmid. The tailed plasmid and cDNA are then annealed to insert the cDNA in the appropriate site of the plasmid and to circularize the hybrid DNA, the complementary character of the tails permitting their cohesion (Figure 1). The resulting recombinant DNA molecule now carries a gene at the chosen PstI restriction site (Figure 1). This method of dA-dT tailing for insertion is described by D.A. Jackson et al., "Biochemical Methods For Inserting New Genetic Information Into DNA Of Simian Virus 40 : Circular SV40 DNA Molecules Containing Lambda Phage Genes And The Galactose Operon Of Escherichia coli", Proc. Natl. Acad. Sci. USA, 69, pp. 2904-2909 (1972) and R. Devos et al., supra. It results in about 3

check has-been?

prepared. Again, only a very few of these clones will contain the gene for FIF or fragments thereof (Figure 1). The preferred host in accordance with this invention is E. coli BH 101.

1. Preparation of PstI-Cleaved, d~~GMP~~A-elongated pBR322

Plasmid pBR322 (~~30 ug~~) was digested ~~with 21 units~~ fully completely with PstI endonuclease (New England Biolabs) at 37°C in ~~150 ul~~ 10 mM Tris-HCl (pH 7.6), ~~6~~ 7 mM $MgCl_2$, ~~50 mM NaCl~~, ~~6~~ 7 mM 2-mercaptoethanol, ~~300 ug/ml of bovine serum albumin ("BSA") (Calbiochem)~~. ~~After 2 h at 37°C~~, the mixture was extracted ~~several times~~ with 1 vol phenol and ~~2.5~~ 10 vol ether and precipitated with 2.5 vol ethanol; 0.2 M Sodium acetate solution

Addition of homopolymeric dA tails (Figure 1) by terminal deoxynucleotidyl transferase (TdT) (purified according to L. Chang and F.J. Bollum, "Deoxynucleotide-Polymerizing Enzymes Of Calf Thymus Gland", J. Biol. Chem., 246, pp. 909-16 (1971)) was done in a 50 ul reaction volume containing 0.14 M potassium cacodylate, 30 mM Tris-HCl (pH 6.8), 1 mM $CoSO_4$, 0.2 ug/ul heat-inactivated bovine serum albumin, 0.8 mM DTT, 0.2 mM dATP and some α^{32}P-dATP. Incubation was at 37°C for 5 min before EDTA was added to 10 mM and SDS to 0.1 % and the mixture extracted with phenol and chromatographed on Sephadex G50 in TE buffer. The void fractions, containing the linearized and elongated pBR322, were further purified by adsorption in 10 mM Tris-HCl (pH 7.8), 1 mM EDTA and 0.4 M NaCl to oligo(dT)cellulose. After extensive washing, the desired fractions were eluted with 10 mM Tris-HCl (pH 7.8) and 1 mM EDTA.

2. Preparation of dT-elongated DNA

Double-stranded DNA was elongated with dTMP residues in similar fashion to that described above for dA tailing of pBR322, except that dTTP and some ^{3}H-dTTP replaced the dATP and α-^{32}P-ATP. Purification on oligo(dT) cellulose was, of course, omitted. As before, the dT-elongated DNA is a mixture of different species, only a very few of which are IF-related (Figure 1).

3. Preparation of Ca^{++}-Treated E.coli HB101

Ca^{++}-treated E.coli HB101 was prepared by the method of

E.M. Lederberg and S.N. Cohen, "Transformation of *Salmonella Typhimurium* By Plasmid Deoxyribonucleic Acid",
J. Bacteriol., 119, pp. 1072-74 (1974) by inoculating the *E.coli* HB101 (a gift from H. Boyer) into 5 ml LB medium (10 parts bacto-tryptone, 5 parts yeast extract and 5 parts NaCl per liter) and cultures grown overnight at 37°C. The fresh cultures were diluted 1/100 in 20 ml LB medium and grown to a density of about 2×10^8 bacteria per ml, quickly chilled in ice and pelleted at 6000 rpm for 5 min in a Sorvall SS34 rotor at 4°C. The cells, kept at 0-4°C, were washed with 20 ml 100 mM $MgCl_2$, repelleted by centrifugation and suspended in 10 ml 100 mM $CaCl_2$. After 20 min in ice, the cells were repelleted and resuspended in 2 ml 100 mM $CaCl_2$ and maintained at 0°C for 15 min. Aliquots (200 µl), supplemented with glycerol to 11%, could be stored for several months at -80°C without loss of activity (D.A. Morrison, "Transformation in *Escherichia coli* : Cryogenic Preservation of Competent Cells", *J. Bacteriol.*, 132, pp. 349-51 (1977)).

4. <u>Annealing of dA-elongated pBR322 and dT-elongated DNA</u>

The vector's and DNA insert's complementary dA- and dT-tails permit annealing to form the desired hybrid plasmid or recombinant DNA molecule. For this purpose, the dA-tailed *Pst*I-cleaved pBR322 vector and the mixture of sized dT-tailed cDNAs were dissolved in TSE buffer (10 mM Tris-HCl (pH 7.6), 1 mM EDTA, 100 mM NaCl) to 1.5 µg/ml plasmid and to a molar ratio of plasmid to DNA insert of 1.5 to 2.0. After heating to 65°C for 10 min, the mixture was cooled slowly to room temperature over 4 h.

The product is, of course, a large mixture of different recombinant DNA molecules and some cloning vehicles without inserted DNA sequences. However, each recombinant DNA molecule contains a cDNA segment at the *Pst*I site. Each such cDNA segment may comprise a gene or a fragment thereof. Only a very few of the cDNA segments code for FIF or a portion thereof (Figure 1). The vast majority code for one of the other proteins or portions thereof whose mRNA's were part of the poly(A) RNA used in the process of this invention (Figure 1).

HB101

5. Transfection Of E.coli ~~X1776~~ With The Annealed Hybrid Plasmids

. P3 containment facilities were used for the transfection process and all subsequent steps in which the resulting transformed bacteria were handled. Aliquots (90 µl or less) of the above mixture were cooled to 0°C and 1 M $CaCl_2$ added to 0.1 M. Aliquots (100 µl or less) of this solution were added to 200 µl Ca^{++}-treated E. coli HB101 in ice and after standing at 0°C for 30 min. the cells were heat-shocked for 5 min at 37°C and cooled again at 0°C for 15 min. After addition of 2 ml LB-medium, the cells were incubated at 37°C in a shaking water bath for 30 to 45 min and the bacterial suspension plated out onto 1.2% agar plates, containing LB medium supplemented with 10 µg/ml tetracycline.

Since plasmid pBR322 includes the gene for tetracycline resistance, E.coli hosts which have been transformed with a plasmid having that gene intact will grow in cultures containing that antibiotic to the exclusion of those bacteria not so transformed. Therefore, growth in tetracycline-containing culture permits selection of hosts transformed with a recombinant DNA molecule or recyclized vector.

After 24 h at 37°C, individual colonies were picked and suspended in 100 µl LB medium (supplemented as above) in the wells of microtiter plates (Dynatech). After incubation at 37°C overnight, 11 µl dimethylsulfoxide were mixed into each well and the trays sealed with adhesive tape. The plates were stored at -20°C and a library of 17,000 individual clones of transformed E. coli HB101 was prepared. This library was derived from 270 fmoles (128 ng) dT-tailed cDNA inserts, which in turn were synthesized from 4.4 µg gradient purified poly(A) RNA. About 98% of the clones of this library (band on representative fractions) were sensitive to carbanicillin (a more stable ampicillin-derivative). Therefore, about 98% of the library contained a plasmid having an insert in the PstI-site of the β-lactamase gene of pBR322 only about 2% contained a recyclized vector without insert.

These 17,000 clones contain a variety of recombinant DNA molecules representing complete or partial copies of the mixture of mRNAs in the poly(A) RNA preparation from FIF-producing cells (Figure 2). The majority of these will contain only a single recombinant DNA molecule. Only a very few of these recombinant

DNA molecules are related to FIF. Accordingly, the clones must be screened to separate the FIF-related clones from the others.

SCREENING FOR A CLONE CONTAINING F IFcDNA

There are several approaches to screen for bacterial clones containing F IF ~~interferon~~ cDNA. ~~("IFcDNA")~~. These include, for example, RNA selection hybridization (Alwine et al., infra), differential hybridization (T.P. St. John and R.W. Davis, "Isolation of Galactose-Inducible DNA Sequences From Saccharomyces Cerevisiae By Differential Plaque Filter Hybridization", Cell, 16, pp. 443-452 (1979)); ~~Hoeijmackers et al., infra)~~, hybridization with a synthetic probe (B. Noyes et al., "Detection And Partial Sequence Analysis Of Gastrin mRNA By Using An Oligodeoxynucleotide Probe", Proc. Natl. Acad. Sci. USA, 76, pp. 1770-1774 (1979)) or screening for clones that produce the desired protein by immunological (L. Villa-Komaroff et al., "A Bacterial Clone Synthesizing Proinsulin", Proc. Natl. Acad. Sci. USA, 75, pp. 3727-31 (1978)) or biologic (A.C.Y. Chang et al., ~~supra~~ "Phenotypic Expression In E. coli Of A DNA Sequence Coding For Mouse Dihydrofolate Reductase", Nature, 275, pp 617-24 (1978)) assays. We have chosen RNA selection hybridization as being the most convenient and promising method for primary screening.

A. RNA Selection Hybridization Assay

1. Overview Of The Initial Assay

Referring now to Figure 2, the recombinant DNA molecules were ~~was~~ isolated from a culture of a mixture of about 46 clones sensitive to carbenicillin and resistant to tetracycline from the above library of clones (two mixtures of 2 clones shown in Figure 2) (Step A). The recombinant DNA molecules were cleaved ~~, denatured~~ and hybridized to total RNA containing F IFmRNA prepared as before (Step B). All recombinant DNA molecule-total RNA hybrids were separated from the non-hybridized total RNA (Step C). The hybridized total RNA was recovered from the hybrids and purified (Step D). The recovered RNA was assayed for F IFmRNA activity as above (Step E). If, and only if, the mixture of recombinant DNA molecules contains a recombinant DNA molecule having an inserted nucleotide sequence capable of hybridizing to the F IFmRNA in the total RNA, under stringent hybridization conditions, will the mRNA released from that hybrid cause the formation of F IF in oocytes, because mRNA released from any other recombinant DNA molecule-total RNA hybrid will not be F IF-related. If a group of 46 clones gave a positive response, the clones were regrouped into 6 subgroups (~~in~~ 4 subgroups ~~lots~~ of 8 and 2 subgroups ~~lots~~ of 7), and

each subgroup assayed as before. This process was continued until a single clone responding to this assay was identified.

There is no assurance that the recombinant DNA molecules and bacterial cultures transformed therewith, which are thus identified, contain the complete F IFcDNA sequence of F IF or even that the DNA sequence actually codes for F IF. However, the recombinant DNA molecules will certainly contain extensive nucleotide sequences complementary to the F IFmRNA coding sequence. Therefore, the recombinant DNA molecule may at least be used as a source of a probe to screen rapidly other recombinant DNA molecules and clones transformed with them to identify further sets of clones which may contain nucleotide an authentic and complete F IF nucleotide coding sequence. The sequence of the inserted DNA fragment of these hybrid plasmids may also be determined and correlated to the amino acid composition and initial sequence reported for authentic F IF (Sug

2. Execution Of The Initial Assay

Step A - Preparation Of The Recombinant DNA Molecule Mixture

Replicas of a microtiter plate containing 96 clones from the above library of clones were made on LB-agar plates, one containing 10 µg/ml tetracycline and the other supplemented with 100 µg/ml carbenicillin. In this manner, two sets of about 45-46 clones, resistant to tetracycline and sensitive to carbenicillin, were picked and grown separately overnight at 37°C in 100 ml LB medium, containing 10 µg/ml tetracycline. These cultures were pooled, spun down in a Sorvall GS-3 rotor at 8000 rpm for 10 min, washed twice with TES buffer (50 mM Tris-Hcl (pH 8), 5 mM EDTA, 5 mM NaCl) and resuspended in 40 ml TES per l of initial culture volume. The cells were lysed with lysozyme-Triton X-100 (M. Kahn et al., "Plasmid Cloning Vehicles Derived From Plasmids ColE1, F, R6K And RK2", in Methods of Enzymology, 68, : Recombinant DNA (R. Wu, ed.) (1980) in press). Forty ml of the TES suspended cells were combined with 20 ml 10% sucrose in 50 mM Tris-HCl (pH 8) and lysozyme to 1.3 mg/ml and allowed to stand at room temperature for 20 min. To this suspension were added 1 ml 0.5 M EDTA-NaOH (pH 8), 8 ml 0.2% Triton X-100, 25 mM EDTA, 50 mM Tris-HCl (pH 8) and the lysis completed at room temperature for 30 min. Cellular debris and most of the chromosomal DNA were removed by pelleting in a Beckmann SW27 rotor at 24000 rpm for 45 min. The supernatant was

R6

cooled in ice, combined with 1/3 vol 40% polyethylene glycol 6000 - 2 M NaCl and allowed to stand overnight at 0°C. The resulting precipitate was collected in a Sorvall HB4 rotor at 5000 rpm for 10 min at 4°C and dissolved in TES buffer. The solution, with 0.2 vol 10 mg/ml ethidium bromide (Serva) and CsCl to 1 g/ml, was centrifuged in a Beckmann R60 Ti-rotor at 40000 rpm for at least 48 h, one polyallomer tube usually being sufficient for the lysate from 1-2 l of original culture volume. Two DNA bands could be visualized in the tube ~~by~~ under UV-illumination. The band of highest density corresponds to plasmid form I DNA, the second band corresponds to form II and form III plasmid DNAs and some chromosomal DNA. The first band was cut from the tube, ethidium bromide removed by six isoamyl alcohol extractions, and the aqueous phase diluted with 3 vol water-supplemented with up to 0.2 M sodium ~~azide~~ acetate (pH 5.1) before DNA precipitation with 2.5 vol ethanol. The DNA was redissolved, extracted with phenol and again precipitated with ethanol. The quality of the DNA was monitored by electrophoresis on a 1% agarose gel in 40 mM Tris-acetate (pH 7.8), 20 mM sodium acetate, 2 mM EDTA, followed by ethidium bromide staining. If the DNA ~~contained~~ was contaminated with form II or form III DNAs, it was further purified by neutral sucrose-gradient centrifugation: 300 µg DNA in 10 mM Tris-HCl (pH 7.6) and 1 mM EDTA was loaded on a 36-ml 5-20% sucrosegradient in 10 mM Tris-HCl (pH 7.6), 1 mM EDTA, 1 M NaCl, centrifuged in polyallomer tubes for 16 h at 24000 rpm in a Beckmann SW27-rotor at 18°C and the DNA containing fractions (OD_{260}) pooled and precipitated with sodium acetate-ethanol.

Step B - Hybridization Of The DNA With Total RNA

About 150 µg DNA, thus prepared, was combined with some uniformly labelled ^{32}P-marker DNA and 2 µg pSTNV-1 DNA (a recombinant plasmid containing a full size cDNA copy of satellite tobacco necrosis virus ("STNV")-RNA; J. Van Emmelo et al., "Construction And Characterization Of A Plasmid Containing A Nearly Full-Size DNA Copy Of Satellite Tobacco Necrosis Virus RNA", J. Mol. Biol., submitted for publication) as internal control, sheared by sonication in an MSE sonicator and precipitated with sodium acetate-ethanol.

A diazobenzyloxymethyl (DBM)-cellulose solid matrix (cf., J.C. Alwine et al., "Method For Detection Of Specific RNAs In Agarose Gels By Transfer To Diazobenzyl Oxymethyl Paper and Hybridization With DNA Probes", Proc. Natl. Acad. Sci. USA, 74,

pp. 5350-54 (1977)) was prepared according to the method of J.C. Alwine et al., "Detection of Specific RNAs or Specific Fragments of DNA ... Method in Enzymology, 68: Recombinant DNA (R. Wu, ed.) (1980) (in press). For a paper matrix, a sheet of Whatman 540 paper was evenly soaked in 2-3 ml 1-(m-nitrobenzyloxy)methyl pyridinium chloride (NBPC/BDH) - 0.7 ml sodium acetate trihydrate - 2.8 ul water per cm^2, incubated at 60°C until dry and for a further 10 min, and baked at 130-135°C for 30-40 min. After washing several times with water (about 20 min), 3 times with acetone (about 20 min) and drying, the paper was incubated at 60°C for 30 min in 0.4 ml 20 % sodium dithionite-water with occasional shaking. The paper was again washed four times with water, once with 30% acetic acid for 5 min and four times with water, transferred for 30 min at 0°C to 0.3 ml per cm^2 ice-cold 1.2 M HCl to which 10 mg/ml fresh $NaNO_2$ had been added immediately before use, and washed twice quickly with ice-cold water and once with 80% dimethyl sulfoxide (spectrophotometric grade, Merck) - 20% 25 mM sodium phosphate (pH 6.0). For a powder matrix essentially the same procedure was followed using microgranular cellulose powder (Whatman CC31), the quantities being expressed against the corresponding weight of the cellulose matrix.

Initially, we used a powder matrix because the capacity for binding was higher, so relatively smaller volumes for hybridization, washes and elution could be used. Subsequently, we used a paper matrix for individual clone screening. Use of paper permits efficient elution with water which proved superior for the later assay of F IFmRNA.

The DNA (50-100 µg (for powder), ~~for paper~~ 3-4 µg (for paper)) prepared above was dissolved in 25 mM sodium phosphate (pH 6.0) heated for 1 min, chilled and four vol DMSO added. Coupling to the matrix (50 mg (powder) or a disc (20 mm dia.) (paper)) usually proceeded over a weekend at 4°C with continuous mixing. The volume of the DNA was kept rather small to allow close contact with the matrix and thereby enhance efficient coupling of the DNA to the matrix. After coupling, the matrix was washed four times with water, and four times with 0.4 N NaOH at 37°C for 10 min each, again four times with water at room temperature and finally twice with hybridization buffer (50 % formamide (deionized, Baker), 40 mM piperazine-N,N'-bis(2-ethane sulfonic acid) (pH 6.4) ("PIPES", Sigma), 1 mM EDTA, 0.6 M NaCl and 0.1 % SDS) at 4°C. Coupling efficiencies were measured by ^{32}P-radioactivity.

Twenty µg total RNA, prepared as before, and 50 ng STNV-RNA were dissolved in 250 µl (50 µl for paper matrix) hybridization buffer and added to the DNA coupled matrix. The matrix was heated to 70°C for 2 min and held at 37°C overnight with gentle mixing.

Step C - Separation Of Hybridized Total RNA-DNA From Non-Hybridized Total RNA

After centrifugation of ~~the above~~ a powder matrix, the unhybridized RNAs were removed and the matrix washed seven times with (0.28 ml) 50% formamide, 10 mM PIPES (pH 6.4), 1 mM EDTA, 0.3 M NaCl and 0.1% SDS, the lower salt content of these washes destabilizing non-specific RNA-DNA binding. Each wash was followed by centrifugation and resuspension of the matrix in the buffer. For subsequent assay, the first wash was pooled with the unhybridized DNA ("Fraction 1") and washes 2-4 ("Fraction 2") ~~as~~ and washes 5-7 ("Fraction 3") were pooled. In ~~these~~ hybridizations to a paper matrix, a similar procedure was ~~emphasized~~ utilized except that 0.1 ml water ~~(0.14 ml)~~ was used for each wash.

Step D - Purification Of Hybridized Total RNA

The hybridized total RNA-DNA was eluted from ~~the~~ a powder matrix with 900 µl 99% formamide, 0.2% SDS at 70°C for 2 min and chilled in ice (A.G. Smith, personal communication). The hybrized total RNA-DNA was eluted from ~~the~~ a paper matrix by first washing with 100 µl of ice cold water and following that two 150 µl water elutions at 80°C for 2 min. For subsequent assay these elutions and the 100 µl wash were pooled ("Fraction 4").

To one-half of each fraction, 0.1 µg calf liver tRNA or ribosomal RNA were added (Fractions 1A, 2A, 3A and 4A) and to the other half 8 µg eukaryotic poly(A) RNA or ribosomal RNA were added (Fractions 1B, 2B, 3B, 4B). The fractions were purified by precipitation by the addition of 0.5 M NaCl and 2.5 vol ethanol to remove traces of formamide and other impurities.

Step E - Determination Of F IFmRNA Activity

Fractions 1A, 2A, 3A and 4A were translated in 25 µl nuclease-

(A)

After incubation, 25 ~~Twenty-five~~ µl ~~rat~~ lymphocyte lysate, from above, were combined with 1 µl 10% deoxycholate - 10% Triton X100 and 2 µl antiserum - PBS (1:9) and incubated at 37°C for 1h. Twenty µl Staphylococcus aureus Cowan I (freshly washed, S. W. Kessler et al., "Rapid Isolation OF Antigens From Cells WITH A Staphylococcal Protein A-Antibody Adsorbent: Parameters of the Interaction of Antibody-Antigen Complexes With Protein A", J. Immunology, 115, pp. 1617-1624 (1975)) in 10% 100 mM NaCl, 10mM Tris-HCl (pH 7.4), 1mM EDTA, 0.05% NP40 were added and the mixture maintained at 20°C for 30 min and centrifuged in an Eppendorf 5412 centrifuge for 2 min. The pellet was washed and centrifuged twice with PBS and the final pellet dissolved in sample buffer and electrophoresed in 13% polyacrylamide ~~gels~~ as described by U.K. Laemmli ~~K.B.~~ ~~et al.~~, "CLeavage of Structural Proteins During The Assembly OF THE Head OF Bacteriophage T4".

Insert A

(prepared according to the procedure of

treated rabbit reticulocyte lysate (R.B. Pelham and R.J. Jackson, "An Efficient mRNA-Dependent Translation System For Reticulocyte Lysates", Eur. J. Biochem., 7, pp. 247-56 (1976)) in the presence of ^{35}S-methionine, ~~immunoprecipitated with antiserum (~~ ~~), electrophoresed in 13% polyacrylamide gel (U.K. Laemmli,~~ Nature, 227, pp. 680-685 (1970), and autoradiographed. Comparison of the STNV-RNA translation products in Fractions 1A and 4A provide an indication of the efficiency of hybridization and RNA degradation in the process. Fractions 1B, 2B, 3B and 4B were dissolved in 2 µl water and assayed in oocytes for F IFmRNA content as described above.

3. Subsequent Assay - Hybridization To Nitrocellulose Sheets

Some subsequent assays of individual clones were done on nitrocellulose sheets (M. Cochet et al., "Cloning Of An Almost Full-Length Chicken Conalbumin Double-Stranded cDNA", "Nucleic Acids Res., 6, pp. 2435-2452 (1979)). The DNA was dissolved in 2M NaCl and 0.2 M NaOH, heated at 100°C for 1 min, chilled, and spotted on detergent free Millipore filters (pore size 0.45 um; 1 mm dia.). The filters were baked for 2 h at 80°C, washed in 0.3 M NaCl, 2 mM EDTA, 0.1% SDS, 10 mM Tris-HCl (pH 7.5) and dried at room temperature. The RNA was hybridized for 3 h at 47°C in 30% formamide, 0.5 M NaCl, 0.4% SDS, 2 mM EDTA, 50 mM PIPES (pH 7.5). Hybridization was stopped by dilution with 10 vol 0.1 M NaCl and the filters were washed several times in 15 ml of 0.3 M NaCl, 0.1% SDS, 2 mM EDTA, 10 mM Tris-HCl (pH 7.5) by shaking at 45°C and several times in the same solution without SDS at 4°C. Elution of the hybridized RNA-DNA was effected in 30 µl 5 mM potassium chloride at 100°C for 1 min.

4. Results Of The RNA Selection Hybridization Assay

Sixteen groups of about 46 clones were screened (Groups A-P). In six of the groups, Fraction 1B contained the only F IFmRNA activity, in eight of the groups no F IFmRNA was detected and in two groups (Groups C and D) F IFmRNA was observed in Fraction 4B. The positive assays are reported in the following format: logarithm of F IF units (calibrated against reference standard 69/19),

Lysates", Biochem. Biophys. Res. Commun., 83, pp. 665-673 (1978).

assay of Fraction 1B (background) and assay of Fraction 4B.

Group	Fraction 1B	Fraction 4B
C	1.0	0
	0.5	0.5
	0	0.2
O	0	0
	0.2	0.5

Group O was subdivided into 6 subgroups (Subgroups O_1 to O_6; (four of eight clones and two of seven clones) and hybridized and assayed as before, except a 400 ml culture per clone was used. The subgroups gave the following results, presented in the same format as above:

Subgroup	Fraction 1B	Fraction 4B
O_1	0	1.2
	0	1.5
	0	0.5
	0	0.5
	0.2	0.5
	0	1.2*
O_2	0.7	0
O_3	0.7	0
	0.5	0
O_4	0	0
O_5	0.5	0
O_6	0	0

* DPM paper method

Subgroup O_1 was subdivided into its individual clones (designated clones ~~1-8~~ $O_{1/1}$ - $O_{1/8}$) and hybridized and assayed as before, except a 700 ml culture per clone was used :

Clone	Fraction 1B	Fraction 4B
$O_{1/1}$	0.2	0
	0.7	0
	0.7	0*
	1.0	0**

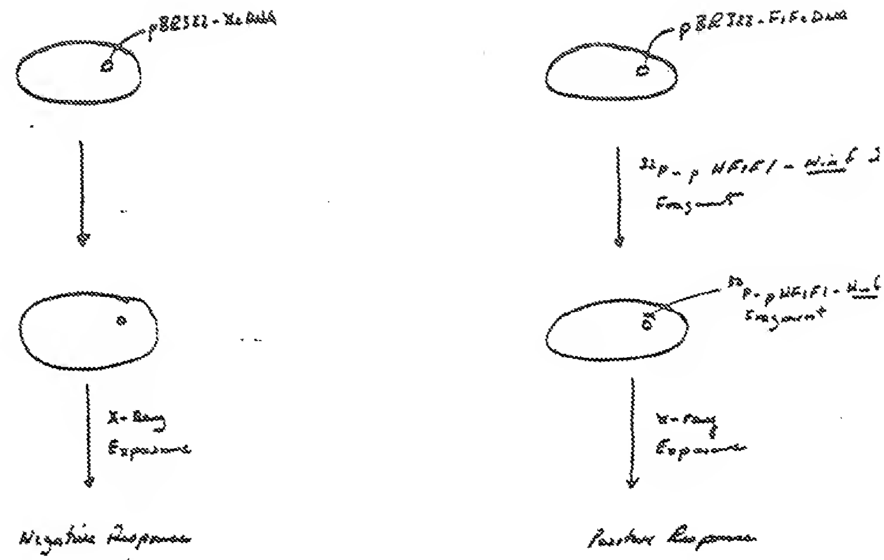
	Fraction 1B	Fraction 4B
$O_{1/2}$	1.2	0
	0.2	0*
	0.7	0**
$O_{1/3}$	1.2	0
	1.0	0.2*
	1.2	1.0(?)*
	1.2	0**
$O_{1/4}$	1.2	0
	1.2	0
	1.0	0*
	1.2	0**
$O_{1/5}$	0.7	0
	0.7	$\leq$ 0.2*
	1.0	0**
$O_{1/6}$	0.7	0
	1.0	$\leq$ 0.2*
	0.5	0**
$O_{1/7}$	0.5	0
	1.2	0*
	< 0.2	0.5**
$O_{1/8}$	0	1.7*
	< 0.2	1.2*
	0	0.7**
	0	1.0**

* DPM paper method

** Nitrocellulose sheets

Therefore, clone $O_{1/8}$ contains a recombinant DNA molecule capable of hybridizing F IFmRNA from total RNA containing F IFmRNA. Non-specific RNA-DNA binding is unlikely, because a comparison of fractions 1A and 4A revealed substantially no non-specific binding of STUV DNA .

Fig. 3



Rik → 1/ digest for sequence
2/

Jan → 1/ clone –
2/

Erik = cl 3 Hya Sm 3A Hinf Sln

→ $\boxed{\text{Hya II}}$ → — toxid
— Bal + toxid

→

AUG

ser + FIF ZO
AB

– Y 3' end 240 nucl
190 aa 570 nucl
Σ 810 nucl.

?
soluble sum
Isolation (zone) + Rel... soluble 4 $(NH_4)_2SO_4$ ✓ dialyse soluble
+ ... + ... constant ...

cl 3
7
II 3-4
SRKB 4 ml
SRKB + FIF
Buffer + FIF
Buffer + ...
800 ml → 4 ml
4 ml

Dec 30 02

... direkt met ...

Alle assays met Penicilline

This is EXHIBIT FIERS-12
to
the Affidavit of Walter C. Fiers
sworn before me
this 19th day of November, 2001

Commissioner for Oath or Notary Public

1 Sequence

Rsa
Hinf
Hpa II
andere ?

2 Restrictiemap   cl 3
+
andere ?

Hpa sites?
EcoRI ?   Pvu I - Pvu II   (welke worden reeds gebruikt
orientatie : Hpa Hin II   of Hpa I - RI
mutant meer clones na screening

3 Expressie (A)   cl 3   betere ... + ...

(B)   ins Hpa II in SRK
werkwijze - ... ... Bel.
orientatie

Opened Dec 30 2002
Décachetée le

Commissioner of Patents
Commissaire des brevets

In presence of examiner
en présence de l'examinateur

I-IF   - detectie I-IF mRNA
- bereiding - RNA uit tonsils
- stimulatie : tonsils + blood buffy coat
+ a buffycoat
- screening   blood lymphocytes : 100 - 500 ... clones

ST NV
F-IF

[illegible]
Laboratorium voor
Moleculaire Biologie
[illegible]

Dec 30 02

[signature]

In presence of / en présence de l'exploitateur [signature]

This is EXHIBIT FIERS-13
to
the Affidavit of Walter C. Fiers
sworn before me
this 19 th day of November, 2001

Commissioner for Oath or Notary Public

exp 128 Ze [illegible] worden [illegible] met
het pHFIF-[illegible] fragment, [illegible]
[illegible] aan [illegible] met IF-RNA.

hybridisatie op filters van DNA-[illegible] (zie exp 126
op het [illegible])

[illegible] met H_2O.

2 filters [illegible] 3 µg DNA

[illegible] DNA pHFIF-2
DNA pHFIF-3

hybridisatie in [illegible]

resultaat na [illegible] 0.5 [illegible] op [illegible].

pHFIF-2	[illegible] 0	[illegible]	[illegible] 0.2	[illegible] 2.2
pHFIF-3	0	0	0	2.5

Dan [illegible] zijn [illegible] effect [illegible] IF-RNA te
hybridiseren.

[illegible] pBR322

[illegible] LB / Tet

[illegible] 1/10 [illegible]

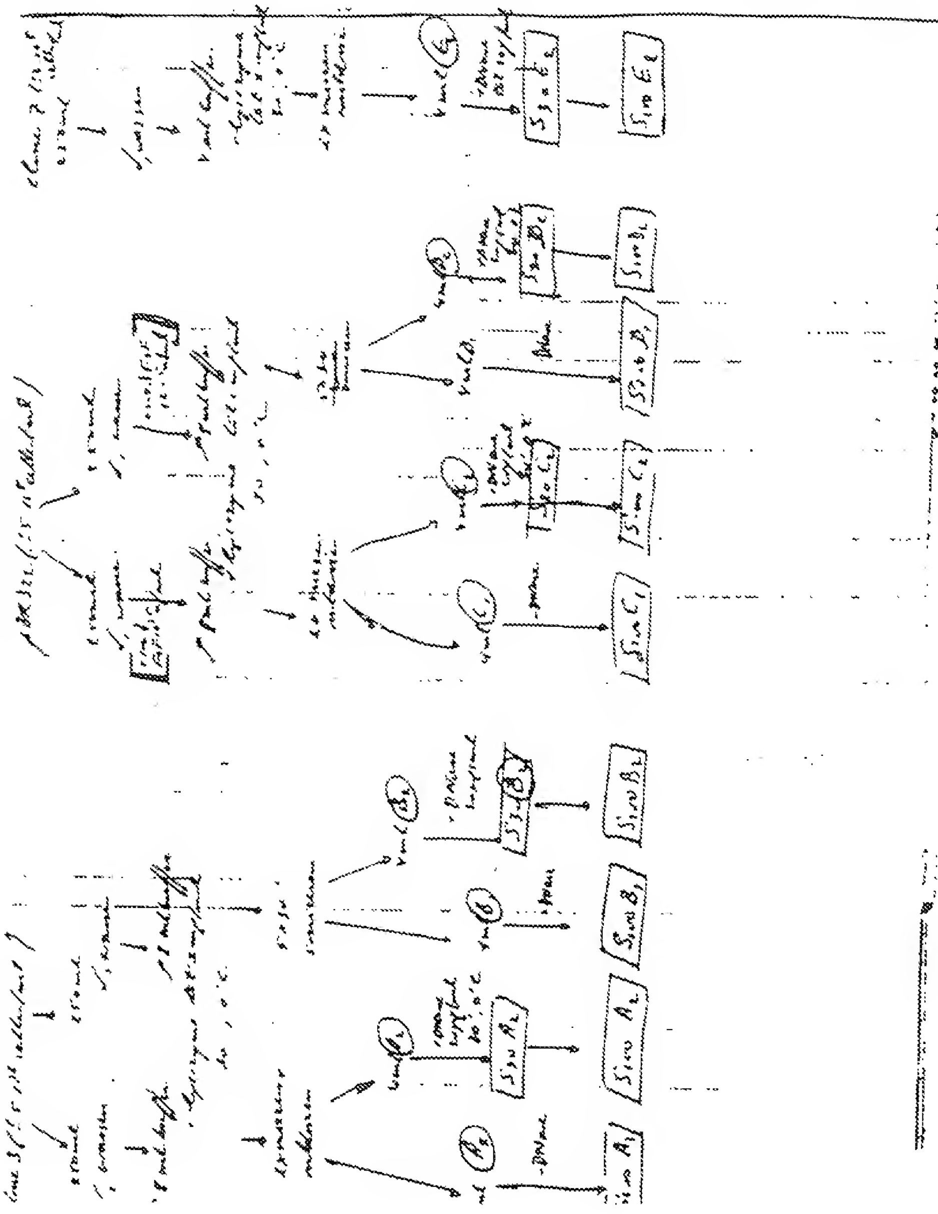
[illegible] 5×10^8 cells / ml.

[illegible]

5 mM Tris pH 8.0

30 mM NaCl

[illegible] 650 nm



[illegible]

[illegible]

[illegible]

A_1	1.2	[illegible]	1.2
A_2	0.7		0.7
B_1	?(5.0.5)		5.0.2
B_2	?(6.0.5)		0.5
C_1	0.7		0.7
C_2	0.7		0.7
D_1	0.7		0.7
D_2	0.7		0.7
E_2	0.7		0.7

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]
het vermoeden dat [illegible] mg [illegible] =
[illegible]
dan de [illegible] mg [illegible]

de [illegible] daarop.

mg neutralisatie bij 10^{-7}
niet meer bij 10^{-8}

Trypsinebehandeling

Sin A, en Sin C,
trypsine behandeling met 0.5 mg/ml [illegible]
[illegible] stoppen met trypsine inhibitor
([illegible])
→ volledige neutralisatie
T.I.

[illegible], enkele kontroles
Sin A, + TI [illegible]
C, + T.I.
IF + TI

dus TI op zichzelf [illegible].

RNase
(is het [illegible] RNA?)
Sin A. RNase [illegible] het [illegible]
[illegible], maar op [illegible]

kontrole poly I:C . 0.4 . [illegible] effect [illegible]
T.I.F. . 0.4 . [illegible]

Sin A, [illegible] op
([illegible])
Tx C_y 5M DAK
C.O.5. #3.0. (?!) C[illegible]

of [illegible] synthetic
([illegible])

[illegible] met de RNA
[illegible]
[illegible] superinductie.
[illegible]
[illegible]

Dus wellicht [illegible] RNA

[illegible]

[illegible] — [illegible] [illegible] [illegible] /
[illegible] $5 \cdot 10^7$ /[illegible]
[illegible] [illegible] [illegible] 37 °C [illegible] F.I.F
- [illegible] [illegible] 37 °C
- $(NH_4)_2SO_4$ / (55% [illegible]) / [illegible] [illegible]
- [illegible] [illegible] [illegible] +3% F.C.S.
[illegible] pH 2 ... HCl [illegible] 10 °C / [illegible] NaOH

(Filtered in [illegible])

[redacted] pRS52-7 ....

→ [illegible] 12.5 ml + 100 µl FIF 10^{-3} [illegible] [illegible] [illegible] — [illegible]
0.7

→ 12.5 ml + 100 µl FIF 10^{-3} [illegible] [illegible] — [illegible]
0.7

→ 12.5 ml + 100 µl F.I.F. [illegible] — [illegible]

* 0.2 — 0.5 ml [illegible]
< 0.2 — 100 µl + AB [illegible]
< 0.2 — 100 µl + [illegible] [illegible] / [illegible] F.C.
≤ 0.7 — 100 µl + [illegible]
[illegible] [illegible] (T.I.)
0.2 — [illegible] [illegible] (Final 100 µl)
[illegible] $(NH_4)_2SO_4$
0.5 — 0.5 ml pH 2 ...
(< 0.5) — 100 µl [illegible] 56 °C

→ 12.5 ml ([illegible] F.I.F.) [illegible]
* 0.7 [illegible] — [illegible]
0.7 [illegible] — 100 µl + [illegible] F 10^{-3} F.C.
0.7 [illegible] — 100 µl + [illegible] F 10^{-3} F.C. + [illegible] A.B.

[redacted] clone 7 [illegible] 12.5 ml — [illegible]

< 0.2 — [illegible]
* 0.7 — 100 µl + AB 0.2 M [redacted]
< 0.2 — 100 µl + AB [illegible]
< 0.7 — 100 µl + AB [illegible]

[redacted] Controls

— 2.5 · [illegible] F 10^{-3} F.C. [illegible] 3% F.C.
0.7 · FIF 10^{-3} F.C. [illegible] 3% F.C.

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

Vgl. Tabel. [illegible]

	VGJ	E, SM	Tx	Primary rabbit kidney PRK	Rabbit kidney RK13	BSC-2	Human [illegible]	[illegible]	Ven	Hal
Leit	3.0	2.5	3.5	1.5	1.0	2.3	~~[illegible]~~ 1.0	3.4	1.8	1.3
K1F	2.4	1.3	3.5	1.5	1.0	2.0	1.5	1.3	1.1	0.0
IIF	2.5	2.8(1)	3.5	0	0	0.8	0	0	1.1	1.0

[illegible]

[illegible]

$(NH_4)_2SO_4$ [illegible] $(NH_4)_2SO_4$ [illegible]

[illegible] PBS

[illegible] PBS

[illegible] HCl

[illegible] PBS

[illegible]

[illegible]

Buffer [illegible] 0°C		0.2
	[illegible]	0.2
Medium [illegible] 0°C		0.5
[illegible]	[illegible]	0.7
Buffer [illegible] 0°C		0.7
[illegible]	[illegible]	0.7

alle 5% ... [illegible]

[illegible]

[illegible] E. 517 . > 1.7

[illegible]

[illegible] E. 517)

[illegible] J.C.

	VG3	[illegible]	[illegible]	Hela	Human kidney	BSC-1	Vero	PKK	RK 13	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	0	[illegible]	[illegible]	0	[illegible]	[illegible]

[illegible handwritten notes]

clone 3
clone 7

[illegible handwritten notes]

[illegible] 0.7

[illegible] [illegible] [illegible]

[illegible] ([illegible])
[illegible] [illegible] [illegible] [illegible] → 0.5
[illegible] [illegible] [illegible] [illegible] → 0.7

[illegible] 0.7
([illegible])
[illegible] [illegible] [illegible] 0.7 → 0.7
[illegible] 0.7 → 0.7

[illegible] [illegible] 0.5

[illegible]

[illegible] [illegible] [illegible]

[illegible] [illegible] [illegible]
[illegible]

[illegible] [illegible] [illegible]

[illegible] [illegible] ([illegible]) 0.5

[illegible] [illegible] : 0.5 → 1.0
[illegible] [illegible] 0.5 → 1.0

[illegible]

[illegible] [illegible]
[illegible]
- [illegible] [illegible] [illegible] [illegible]
- [illegible] [illegible] [illegible]
- [illegible] [illegible] [illegible] ([illegible])
- [illegible] [illegible] [illegible] [illegible]

- [illegible] [illegible] [illegible]

[illegible] [illegible]
- [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible]
[illegible]
- [illegible] [illegible] [illegible] [illegible] [illegible] [illegible]

[illegible] [illegible]
[illegible] [illegible]

[illegible]

[illegible] ([illegible])

- [illegible]
  [illegible]

- [illegible] 37°C
  [illegible]

[illegible]

- [illegible]
  [illegible]

[illegible]

- [illegible]
  ([illegible])

[illegible]

[illegible]

- [illegible] 0.2 → 0.2

- [illegible] 0.2 → 0.7 ([illegible])

- [illegible]

[illegible] 0.7 → 0.5

[illegible]

[illegible] E.507

[illegible]

JC

[illegible]

			ref. T21	ref. E.507
1/	[illegible]	pH 8	0.7	2.7
2/	idem	pH 7	2.0	2.7
3/	idem	pH 6	0.7	2.7
4/	[illegible]	pH 8	<0.2	<0.2
5/	idem	pH 7	<0.2	<0.2
6/	idem	pH 6	<0.2	1.2(?)
7/	[illegible]	pH 7	<0.2	1.2(?)
8/	[illegible]	pH 7	<0.2	1.2(?)

[illegible] pH 7 [illegible]

[illegible] E.507.

[illegible] JC

[illegible]

[illegible] E.DC [illegible]

[illegible]

T[illegible] [illegible] E.S[illegible]

[illegible]

	T[illegible]	E.S07
Conditions Salmonella [illegible]	[illegible]	[illegible]
E[illegible]	0.3	0.6
Z[illegible]	[illegible]	[illegible]
[illegible] C	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]

[illegible] 132 — [illegible]

[illegible] J.C. [illegible]

[illegible] E, ST [illegible]

[illegible]

[illegible]

C. [illegible]

[illegible] (… T. 10 …)

[illegible]

[illegible]

[illegible] J.C.

	[illegible] E, ST	
	[illegible]	[illegible]
[illegible]	[illegible]	< 0.7
[illegible]	< 0.7	< 0.7
[illegible]	< 0.7	< 0.7
~~[illegible]~~		
[illegible]	0.7	< 0.7
[illegible]	[illegible]	2.0
[illegible]	[illegible]	[illegible]

NB [illegible]

[illegible] T2 [illegible]

[illegible]

[illegible] (…)

[illegible] F.T.

Test 1.C [illegible] op [illegible] synthetase (E. 5[illegible])

	zonder [illegible]	met [illegible]
- cel 6	+ + +	+ +
- pTZ17	+ +	—

[illegible] bij pTZ17. de activiteit [illegible] op [illegible] onderdrukt [illegible]

cel 6 — de [illegible] activiteit van pTZ17 [illegible] dat [illegible] blijft [illegible] Normaal dat het [illegible] onderdrukt.

Dus [illegible] op:
Er is toch iets dat cel 6 onderscheidt van pTZ17
Er [illegible] gemaakt worden dat het synthetase [illegible]?

Eventueel [illegible] op [illegible] op E. 5[illegible]

~~[illegible]~~ [illegible]

Andere stalen

	zonder [illegible]	met [illegible]
- pTZ17 - [illegible]	+ +	+ +
	de activiteit blijft	
- pTZ17 - [illegible]	onduidelijk	+ +
	[illegible]	

[illegible]?

[illegible]